CHARACTERIZATION OF THE ROLES OF RIN AND NOR DURING TOMATO (SOLANUM LYCOPERSICUM) FRUIT RIPENING

by Catherine Martel

CHARACTERIZATION OF THE ROLES OF RIN AND NOR DURING TOMATO (SOLANUM LYCOPERSICUM) FRUIT RIPENING

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Catherine Martel

August 2010

CHARACTERIZATION OF THE ROLES OF RIN AND NOR DURING TOMATO (SOLANUM LYCOPERSICUM) FRUIT RIPENING

Catherine Martel, Ph. D.

Cornell University 2010

Fruit ripening is a complex developmental process involving the precise coordination of multiple physiological and chemical changes, and resulting in the transformation of the seed receptacle into a palatable organ. Numerous studies have demonstrated the role of ethylene as a key regulator of ripening in climacteric species. Whereas numerous ripening-associated traits have been shown to be influenced by ethylene, the characterization of the *rin* and *nor* tomato mutants have uncovered another layer of regulation acting upstream of ethylene. The fruits of these mutants are characterized by an absence of a ripening-associated ethylene burst, and an inability to ripen in the presence of exogenous ethylene. This phenotype is described as a failure to reach ripening competency, a developmentally regulated stage in which a fruit becomes responsive to ethylene. The genes underlying the *rin* and *nor* mutations have been cloned and shown to encode transcription factors of the MADS-box and NAC domain families, respectively. This dissertation uses several molecular approaches to gain insight into the role of these transcription factors during ripening. Antibodies specific for both the RIN and NOR proteins were developed and used to examine the dynamics of protein accumulation during ripening. A chromatin immunoprecipitation approach was used to address the transcriptional regulation of known ripening-associated genes by RIN and NOR.

BIOGRAPHICAL SKETCH

Catherine Martel was born in Greenfield Park, Canada, on January 10th 1978. She completed a B.Sc degree in Biochemistry at the University of Montreal in May 2000. She obtained a M.Sc in Molecular Biology at the University of Montreal in May 2003 during which she studied the role of the RNA binding protein Staufen in mammals. Her interest for plant biotechnology led her to enroll in the PhD program Plant Cell and Molecular Biology at Cornell University in the Fall of 2004.

ACKNOWLEDGMENTS

I would first like to thank my PhD advisor, Pr. James J. Giovannoni, for the help, ideas and advices he provided me with during my doctoral studies. I would also like to acknowledge my committee members, Pr. Gregory B. Martin and Pr. Jocelyn K. Rose, for their suggestions and ideas.

I would like to thank all my colleagues from the Giovannoni lab. I really enjoyed working with everybody in the lab during these last 6 years. In particular, I would like to thank my bench-mate Ryan for being such a great friend, inside and outside the lab, and my office-mate, Nigel, for the numerous interesting conversations (not always scientific) I had with him. I would like to thank Julia for sharing her experience and ideas with me and Ruth for always being there to make sure everything runs smoothly in the lab.

I would like to thank all my friends who have made my stay in Ithaca such a unique experience. In particular, I want to thank Janelle and Sarah for being such great friends; and my boyfriend Pat for the support and encouragements he gave me toward the end of my dissertation,. I would also like to thank my parents, Louise and Jacques, for the support and encouragements they gave me throughout my studies.

My work was founded in part by the Government of Canada through an NSERC PhD grant.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 INTRODUCTION

Ripening, the phenomenon, responsible for the producing fruit that are attractive for consumption , has long been a major area of interest due to in part to the important place fruit occupy in the human diet. Ripe fruits are a great source of energy, minerals, vitamins, carotene and antioxidants. Ripening is studied not only for its role in nutrition, but also since it represents a unique and complex developmental process requiring the well-coordinated regulation of numerous biochemical pathways. Study of fruit ripening is therefore valuable not only for practical agricultural purposes but also to better understand the regulation and orchestration of plant developmental programs.

1.1 FRUITS AND RIPENING

1.1.1 Characteristics and definitions

Fruits, unique to angiosperms, are botanically defined as the seed receptacles that develop from the ovary walls of fertilized carpels (Seymour, 1993). Whereas this definition accurately describes fruits such as tomato (*S. lycopersicum*), melon (*C. melo*) and peaches (*P. persica*), extension of this classical definition to include fruits derived from extracarpellary (ie non-ovary derived) tissues is needed to include fruits like apples (*M. domestica*), strawberry (*F. ananassa*) and pineapple (*A. comosus*) (Giovannoni, 2001). Fruits are classified as being either dry or fleshy depending on the nature of the structure containing the seeds. *Arabidopsis thaliana* produces dry siliques, whereas tomato fruits are fleshy. Fleshy fruits are believed to have evolved as a mean to promote seed dispersal by animals in areas where abiotic vectors, such as

wind, were insufficient or of less reliable quality (Seymour G.B, 1993; Seymour, 1993; Giovannoni, 2001).

1.1.2 Tomato as model organism to study fruit biology

Among the numerous species producing fleshy fruits, tomato has emerged as one of the best model systems to dissect the molecular mechanisms underlying development and ripening. Today, tomato represents the seventh most important crop produced in the U.S.A., and the 13th worldwide with a global market value of more than USD $30 billion (FAOstat, www.faostat.fao.org). Increasing importance of tomato fruit in the human diet is but one of the numerous characteristics that render this species attractive for fruit-related studies. Practical advantages of using tomato as an experimental model organism include a short generation time, a newly released genome sequence (http://solgenomics.net), a large expressed sequence tag (EST) collection, its ease of sexual hybridization, year-round growing potential in greenhouses and efficient transformation, allowing for rapid generation of transgenic plants for functional analyses (Fei et al., 2004; Mueller et al., 2005). The long domestication and breeding history of tomato have also resulted in a large germplasm collection, further complemented by several mutagenized populations and introgression lines that can be used to dissect numerous molecular mechanisms involved in plant growth, development and environmental responses (Tanksley and McCouch, 1997; Bai and Lindhout, 2007).

1.1.3 Tomato fruit development

Tomato fruit development can be divided in four distinct phases (Figure 1-1). The first phase, termed the fruit set phase, corresponds to the stage where the development of the ovary either proceeds or aborts. The initiation of ovary development normally depends on the success of pollination and fertilization. Gibberellins and auxins have

been shown to play an important role in fruit set, as application of these hormones to tomato carpels has been shown to trigger the development of parthenocarpic (seedless) fruits (Gustafson, 1960; Gillaspy et al., 1993) .

The second phase of fruit development is characterized by a period of extensive cell division of the fruit tissue that generally lasts between seven and ten days after fertilization depending on genotype (Varga and Bruinsma, 1966; Mapelli and Lombardi, 1982; Bohner and Bangerth, 1988). Most fruit growth is associated with the subsequent expansion phase during which cells undergo substantial endoreplication resulting in the production of large high ploidy cells with a diameter of more than 0.5mm and up to 512C DNA content (Cheniclet et al., 2005). This growth phase is driven by the accumulation of water in the vacuole. Some controversy exists as to the nature of the vascular elements responsible for water import into the fruit tissue. Several studies indicate that the majority of water import is mediated by the phloem rather than the xylem (Ehret and Ho, 1986, 1986; Ho et al., 1987; Plaut et al., 2004; Guichard et al., 2005), but recent papers argue the opposite (Van Ieperen et al., 2003; Windt et al., 2009). Regardless of the underlying mechanism, the developing fruit act as a major sink during this period (10dpa to 35dpa), importing water, sucrose, amino acids and organic acids from the rest of the plant (Ho et al., 1987). Interestingly, the fruit themselves, although photosynthetically active, contribute little to their pool of photoassimilates (Gillaspy et al., 1993). At the end of the cell expansion period, the tomato fruit reaches its final size and contains mature seeds that are ready to be dispersed. The mature fruit then undergoes the last phase of fruit development, known as ripening.

1.1.4 Fruit ripening

The developmental process referred to as ripening is the sum of biochemical and structural changes responsible for converting the fleshy seed receptacle into a palatable tissue attractive to seed-dispersing organisms. Although the exact nature of fruit modifications associated with ripening can vary depending on the species, it generally includes the following changes : modification of cell wall ultra structure, transition of plastids from chloroplasts to chromoplasts, conversion of starch to sugars, increased susceptibility to post-harvest pathogens, changes in the biosynthesis and accumulation of pigments, and changes in the production of aroma- and flavor-associated volatile compounds (Seymour G.B, 1993; Seymour, 1993; Giovannoni, 2001).

Examination of the ripening mechanisms of different species has lead to the subdivision of fleshy fruits into two classes based on ripening physiology: climacteric and non-climacteric fruits. Climacteric fruits (such as tomato, cucurbits, avocado, banana and stone fruits) are characterized by a burst in ethylene biosynthesis at the onset of ripening, quickly followed by an increase in respiration rate principally associated with increased flux through the glycolytic pathway (Seymour, 1993). Climacteric fruits show a complete dependence upon ethylene for both initiation and completion of their ripening program (Millerd et al., 1953). Non-climacteric fruits (such as strawberry, grape and citrus) do not show a ripening-associated increase in respiration and do not produce nor require ethylene to initiate and maintain their ripening program. Recent data however, suggest that a small increase in ethylene production might be important to regulate ripening in these fruits (Trainotti et al., 2005) Another interesting difference between climacteric and non-climacteric fruits is their dependence upon the plant to complete their ripening program. Climacteric fruit have been said to be self-sufficient (Prasanna et al., 2007) in that they can ripen even

if detached from the plant. Conversely, ripening in non-climacteric fruits will slow or even stop ripening if harvested and therefore generally need to remain attached to the plant until at least the initiation of ripening to complete their maturation. Evidently, the downstream mechanisms regulating climacteric and non-climacteric fruit ripening have diverged during evolution of different fruit-bearing species. It is however believed that the upstream, developmentally -controlled, regulatory switches involved in triggering the ripening program are shared between these two classes of fleshy fruit and could even be analogous to the mechanisms involved in dry fruit developmental programs. All of these modifications increase seed-dispersers attractiveness for ripe fruit.

1.1.5 Tomato fruit ripening

In order to better dissect tomato ripening, this process has been further subdivided in several key stages illustrated and summarized in Figure 1-1 and Table 1-1, respectively . Briefly, the mature green (MG) stage refers to the final-sized fruit containing mature seeds before the initiation of ripening. The breaker stage (BK) corresponds to the beginning of the ripening program in the fruit, and is characterized by the first visual sign of ripening (orange color at the base of the fruit) and production of high levels of ethylene. Numerous structural genes associated with ripening begin to be expressed at high level at this stage (Alba et al., 2004; Fei et al., 2004). The red ripe fruit stage (RR) corresponds to the fruit having completed the ripening program and possessing all the characteristics required for its consumption by seed dispersers. In tomato, the time required to reach RR from BK is typically three to ten days, depending on the cultivar.

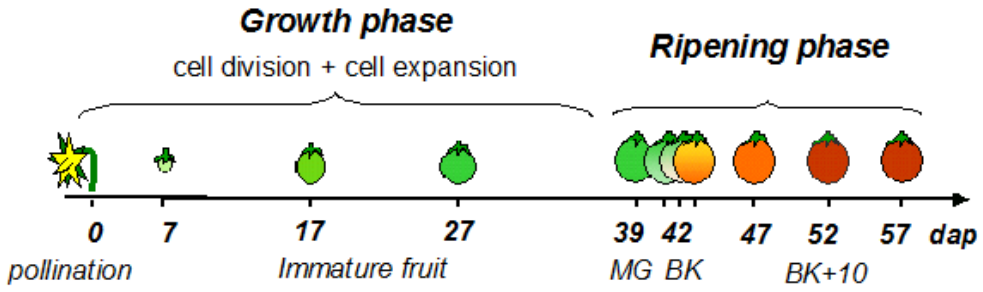


Figure 1-1 Phases of fruit development. Scheme illustrating the different developmental stages of tomato fruit (cv *Ac*) from the time of pollination of the flower to ripe fruit. MG : mature green; BK : breaker, dap : days after anthesis. Figure adapted from Alba et al. (2005).

Table 1-1 Key tomato ripening stages. Stages are identified as MG : Mature Green, BK : breaker, RR : red ripe

Stage	Time (dap)	Characteristics
MG	39	Mature fruit Ripening not initiated
BK	42	Ethylene burst Climacteric respiration Change of color
RR	45-52	Fully ripe fruit

1.2 MECHANISMS REGULATING RIPENING

1.2.1 Ethylene

One of the most striking features of climacteric fruit ripening is the dramatic increase in ethylene production occurring at the onset of ripening. It is therefore not surprising that many prior ripening-related studies have focused on this phenomenon. Numerous studies in both tomato and *A. thaliana* have lead to the elucidation of the signalling pathway and cellular mechanisms responsible for ethylene action in climacteric fruits

(Figure 1-2) (Wilkinson et al., 1997; Bleecker and Kende, 2000; Stepanova and Ecker, 2000).

Ethylene is synthesized from the methionine-derived compound S-adenosyl-methionine (SAM) through the sequential action of two enzymes: ACC synthase (ACS) which catalyses SAM conversion to 1-aminocyclopropane-1-carboxylic acid (ACC) by before being oxidized to ethylene by ACC oxidase (Adams and Yang, 1977). The rate of ethylene synthesis in most plant tissues is limited by available ACS activity (Alexander and Grierson, 2002). This is not necessarily the case in mature tomato fruit where both ACS and ACO are limiting and induced during ripening (Alexander and Grierson, 2002). Numerous members of *ACS* and *ACO* gene families have been described in tomato, some of which are expressed in a fruit specific manner (Rottmann et al., 1991; Barry et al., 2000).

Ethylene perception is mediated by ER–bound receptors termed ETR (*ethylene response*), receptors which resemble bacterial two-component histidine kinases (Chang et al., 1993). ETRs are negative regulators of ethylene signalling and dominant mutations leading to ethylene insensitivity have been shown to result from the constitutive activity of mutant receptors (Chang et al., 1993). The binding of an ethylene molecule deactivates the receptor and induces its rapid turnover - likely through a ubiquitin proteasome-dependant pathway (Kevany et al., 2007). In the absence of ethylene, ETR receptors activate the downstream signalling component CTR1. CTR1 (*constitutive triple response1*) is a member of the raf-like protein kinases and also acts as a negative regulator of the signalling cascade, possibly through a putative MAPKKK activity (Clark et al., 1998; Gao et al., 2003).

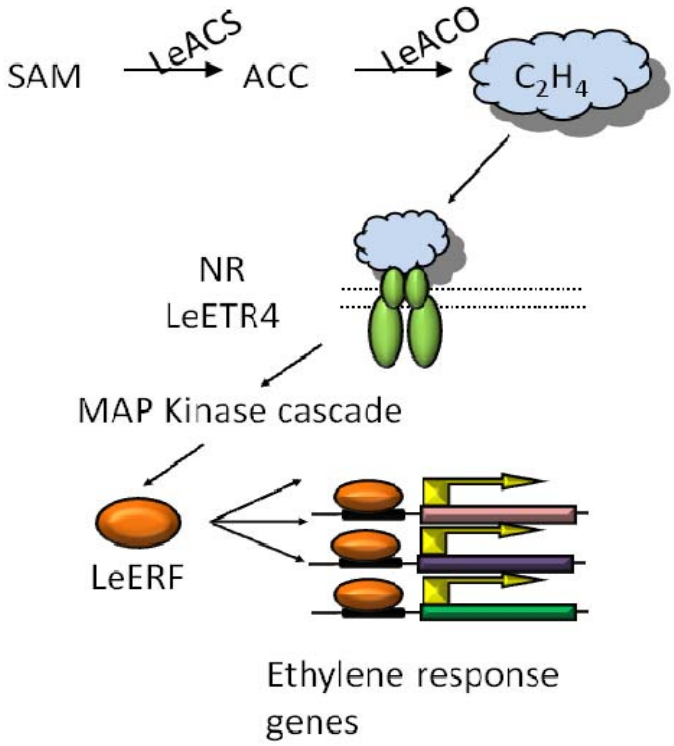


Figure 1-2 Ethylene (C_2H_4) synthesis and signaling cascade in tomato ripening fruits. Synthesis: ACC synthase (LeACS) enzyme converts S-Adenosyl-Methionine (SAM) to 1-aminocyclopropane-1 carboxilic acid (ACC). ACC is oxidized to ethylene (C_2H_4) by ACC oxydase (LeACO). Perception: the membrane-bound receptors NEVER RIPE (NR) and ETR4 bind to ethylene. Signaling: Ethylene binding leads to the expression of ETHYLENE RESPONSE FACTORS (LeERF). LeERF regulate expression of ethylene response genes.

The exact mechanism by which CTR1 negatively regulates the next component of the system, EIN2, remains elusive. EIN2 (*ethylene insensitive 2*) is a membrane-bound protein that shares some similarity with Nramp metal-ion transporters (Alonso et al., 1999; Thomine et al., 2003). It plays an essential role as an integrator of ethylene and other signalling cascades and is, by an as yet undefined mechanism, responsible for the stabilization of the nuclear transcription factor EIN3 (Chao et al., 1997). EIN3 (*ethylene insensitive 3*) is constitutively expressed but rapidly degraded by the

ubiquitin-proteasome pathway, and its stabilization by EIN2 is necessary for efficient binding and transcription of the *ERF1* gene. *ERF1* (*ethylene response factor 1*) encodes a secondary transcription factor that is in turn responsible for the transcriptional regulation of numerous other ethylene responsive genes (Solano et al., 1998).

1.2.2 Ethylene signalling in tomato

The ethylene signalling pathway is well-conserved between *A. thaliana* and tomato. Tomato homologs of the different biosynthesis and perception components have been identified (Lin et al., 2009). Table 1-2 lists *A. thaliana* ethylene components along with their tomato counterparts. As indicated, tomato often possesses many homologous genes encoding each specific component of the pathway. This duplication has allowed sub- and neo-functionalization of ethylene responses to particular situations, one of which being fruit ripening. Genes specifically involved in ethylene signalling during fruit ripening are shown in bold in Table 1-2.

An important feature of ethylene response occurring during climacteric fruit ripening is the switch from an auto-inhibitory response (system 1) to an autocatalytic response (system 2) (Lelievre et al., 1997) . Ethylene production in vegetative tissues and immature fruits is controlled by a negative feedback loop that results in reduction of ACS and ACO activities following exposure to ethylene (Lelievre et al., 1997). This down-regulation is the result of both transcriptional and post-translational (Yoshida et al., 2006) regulations of the ethylene biosynthetic genes (system 1). Interestingly, in ripening fruits and in some senescing flowers, ethylene perception instead triggers an autocatalytic positive feedback loop resulting in a dramatic increase in the rate of ethylene synthesis (system 2). This transition from system 1 to system 2 is necessary for the large ethylene production associated with initiation of climacteric fruit

ripening. Specific members of the LeACS and LeACO families are responsible for this fruit specific response (Nakatsuka et al., 1998; Barry et al., 2000); however the exact mechanism by which these genes became responsive to ethylene at the onset of ripening remains unclear. The existence of this transition at the onset of the ripening program highlights the existence of developmentally-regulated signals acting before the ethylene burst.

Table 1-2 Ethylene signaling components in tomato. Genes in bold have been implicated in fruit ripening

Name	Function	Tomato genes	References
ACC synthase	Ethylene production	**LEACS1A**, LEACS 1B, **LEACS 2**, LEACS3, **LEACS 4,** LEACS5, **LEACS 6**, LEACS7	(Olson et al., 1991; Rottmann et al., 1991; Lincoln et al., 1993; Oetiker et al., 1997; Barry et al., 2000)
ACC oxidase	Ethylene production	**LE ACO1**,LEACO2, **LEACO3, LEACO4**	(Blume and Grierson, 1997; Barry et al., 2000)
ETR	Ethylene receptors	LeETR1,LeETR2, **NR**, **LeETR4**, LeETR5, LeETR6	(Wilkinson et al., 1995; Payton et al., 1996; Zhou et al., 1996; Tieman and Klee, 1999)
CTR1	MAPKKK	**LeCTR1,** LeCTR3, LeCTR4	(Adams-Phillips et al., 2004)
EIN2	Signal transduction	**LeEIN2**	(Wang et al., 2007)
EIN3	TF	LeEIL1, LeEIL2, LeEIL3, **LeEIL4**	(Tieman et al., 2001; Yokotani et al., 2003)

1.2.3 Ripening competency

As exemplified by the transition from system 1 to system 2, the acquisition by mature fruit of a competency to ripen represents the initial physiologically defined step of ripening. The notion of ripening competency is further illustrated by the existence of specific tomato mutants. The fruits of the tomato mutants *ripening inhibitor (rin)* and *non-ripening (nor)* are unable to ripen and lack most phenotypic signs of ripening; remaining green and firm for an extended period of time compared to normal fruits (Figure 1-3; (Vrebalov et al., 2002; Giovannoni, 2007). These mutant fruits also fail

to produce the typical burst in ripening-associated auto-catalytic ethylene production . However lack of ethylene production is not the cause of the absence of ripening since exogenous ethylene does not rescue their non-ripening phenotype. The observation that ethylene-dependent transcription occurs following exogenous ethylene application however indicates that ethylene perception and signalling are functional in these mutants. Thus *rin* and *nor* mutations seem to specifically affect the ability of the fruit to both produce and correctly respond to ethylene. The genes affected by these mutations likely encode proteins that are required for the correct establishment of ripening competency. *RIN* and *NOR* genes encode transcription factors suggesting they might play a coordinating role in both the ethylene-independent and ethylene-dependent regulation of fruit ripening (Vrebalov, pers. comm.; Vrebalov et al. (2002)

Figure 1-3 Phenotype of the *rin* and *nor* mutants. Tomato fruits for wt (cv Ac), *rin* and *nor* mutant plants collected at the BK +7 days stages.

1.3 TRANSCRIPTION FACTORS INVOLVED IN TOMATO RIPENING COMPETENCY ACQUISITION

1.3.1 MADS box

1.3.1.1 *Description*

The *RIN* gene, located on tomato chromosome 5, is a member of the MADS box family of transcriptional regulators (Riechmann and Meyerowitz, 1997). *MADS* box genes encode DNA binding proteins involved in many developmental processes in yeast, insects, nematodes, lower vertebrates, mammals and plants (Theissen et al., 2000; Becker and Theissen, 2003; Messenguy and Dubois, 2003). The name MADS is an acronym of the name of the four founding members of the family: *Mcm1* from *S. cerevisiea* (Passmore et al., 1989), *AGAMOUS* from *A. thaliana* (Yanofsky et al., 1990), *DEFICIENS* from *A. majus* (Sommer et al., 1990) and *Serum Response Factor* (*SRF*) from *H. sapiens* (Norman et al., 1988). Before the divergence of animals and plants, the ancestral MADS box gene is believed to have undergone a duplication event leading to the creation of the two family of MADS box currently recognized: MADS box type I and type II (Shore and Sharrocks, 1995; Alvarez-Buylla et al., 2000). Whereas animals contain functional members of both types, the majority of MADS box genes characterized in plants are more similar to type II (Messenguy and Dubois, 2003). Plants MADS box genes further possess a unique C-terminus region, named the IKC region, not found in other organisms and so are said to belong to MIKC-type of MADS box. MIKC-type MADS box can further be subdivided into two types based on the structure of their I domain, the MIKC*-type and MIKC^{c} –type (Henschel et al., 2002; Parenicova et al., 2003; Kaufmann et al., 2005; Hileman et al., 2006). Almost all MADS box genes that have been functionally characterized belong to the latter type, consequently the term MIKC will be used to refer to MIKC^{c}-type

genes. MIKC-MADS box genes have been involved in the regulation of numerous biological processes in plants, including regulation of flowering time (Michaels and Amasino, 1999; Sheldon et al., 1999; Hartmann et al., 2000; Lee et al., 2000; Sheldon et al., 2000), establishment of meristem and floral organ identity, and differentiation of roots, fruits, leaves and ovules (Riechmann and Meyerowitz, 1997; Theissen et al., 2000; Becker and Theissen, 2003)

1.3.1.2 *Structure of MADS proteins*

As their name implies, MIKC-MADS box proteins are composed of 4 modular domains, namely the M, I, K and C domain, each performing a specific molecular function (Krizek and Meyerowitz, 1996). Figure 1.4 illustrates the structure of a typical MIKC-type MADS box protein.

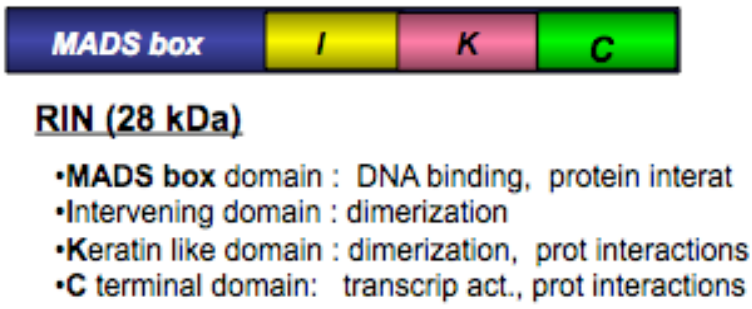


Figure 1-4 Structure of plant MIKC-MADS box protein

The most N-terminal region of the protein, extending between 58 and 60 amino acids, is called the MADS domain, or M, and is the most highly conserved domain of the MADS box protein (Ng and Yanofsky, 2001). It is involved in DNA binding and, specifically recognizes a *cis*-element termed the CArG box (Alvarez-Buylla et al., 2000). The Intervening, or I, domain, whose sequence is generally not highly

conserved among members, has been shown to be important for dimerization of the protein and is also believed to influence DNA binding specificity (Riechmann et al., 1996). The K domain, so called because of its high similarity with the coil-coiled domain of the keratin protein, has been shown to play a role in protein-protein interactions (Ma et al., 1991; Pellegrini et al., 1995; Riechmann et al., 1996; Yang et al., 2003; Yang and Jack, 2004; Immink et al., 2009). The C domain (so named because of its C-terminal location) shows the highest degree of diversity within the MADS box family (Alvarez-Buylla et al., 2000). This domain as been shown to contain motifs involved in transcriptional activation, post-translational modifications and protein interactions (Kramer et al., 1998; Cho et al., 1999; Kramer et al., 1999; Yalovsky et al., 2000; Honma and Goto, 2001; Pelaz et al., 2001; Lamb and Irish, 2003).

Plant MADS box proteins have been demonstrated to bind DNA as hetero- and homodimers (Pellegrini et al., 1995; Tan and Richmond, 1998). MADS box protein dimers recognize and bind to the consensus sequence composed of the nucleotide sequence $CC(A/T)_6GG$, termed the CArG box (West et al., 1998; Egea-Cortines et al., 1999; Tang and Perry, 2003).

1.3.1.3 *MADS Phylogeny*

Thirty nine *MADS* box genes have been described in *A. thaliana* (Parenicova et al., 2003; Kaufmann et al., 2005), while Hileman et al. (2006) and others have identified 36 MADS box genes in *S. lycopersicum*. Depending on the method of phylogenic analysis, the plant MADS box genes can be divided in 11 or 13 subfamilies. The phylogenic tree reproduced in Figure 1-5 illustrates the relationship between the MADS box in *A. thaliana* and *S. lycopersicum* (Hileman et al., 2006). Based on this phylogeny, the *RIN* gene is part of the *SEP* lineage of MADS box family which have

been shown to play key roles in the regulation of flower organ identity in *A. thaliana*. A key feature of this subfamily is their ability to interact with MADS box proteins from other subfamilies to mediate the formation of higher order protein complexes (Fan et al., 1997; Honma and Goto, 2001; Ditta et al., 2004).

1.3.1.4 *ABCE model of floral organ identity*

In order to better understand the function of the *SEP* genes it is worth describing the genetic regulation of floral development in more detail. The involvement of MADS box proteins in the specification of floral organs illustrates their combinatory mode of action. The ABC model (Honma and Goto, 2001) explains the specification of the four floral organs found in angiosperms by the combined activity of A, B and C-class genes. According to this model, the development of each floral organ is the result of the pattern of expression of each gene class in the developing floral meristem. Sepals are the result of the activity of A class genes alone, petals are defined by the combination of A and B class genes, stamens are defined by the simultaneous activity of B and C class genes and carpels develop as a result of C class gene activity alone (Bowman and Meyerowitz, 1991; Coen and Meyerowitz, 1991). Interestingly, the genes responsible for the A, B and C activities all encode MADS box proteins, with the exception of the A class gene AP2 which is a member of the AP2/ERF family of transcriptional regulators. As predicted by the model, each gene is specifically expressed in the floral whorl, where its activity is expected (Riechmann and Meyerowitz, 1997; Davies et al., 1999; Chen, 2004; Robles and Pelaz, 2005). Physical interactions between the ABC MADS box proteins have been demonstrated by yeast-two-hybrid and *in vitro* binding assays (Fan et al., 1997; Riechmann and Meyerowitz, 1997; Egea-Cortines et al., 1999; Pelaz et al., 2001).

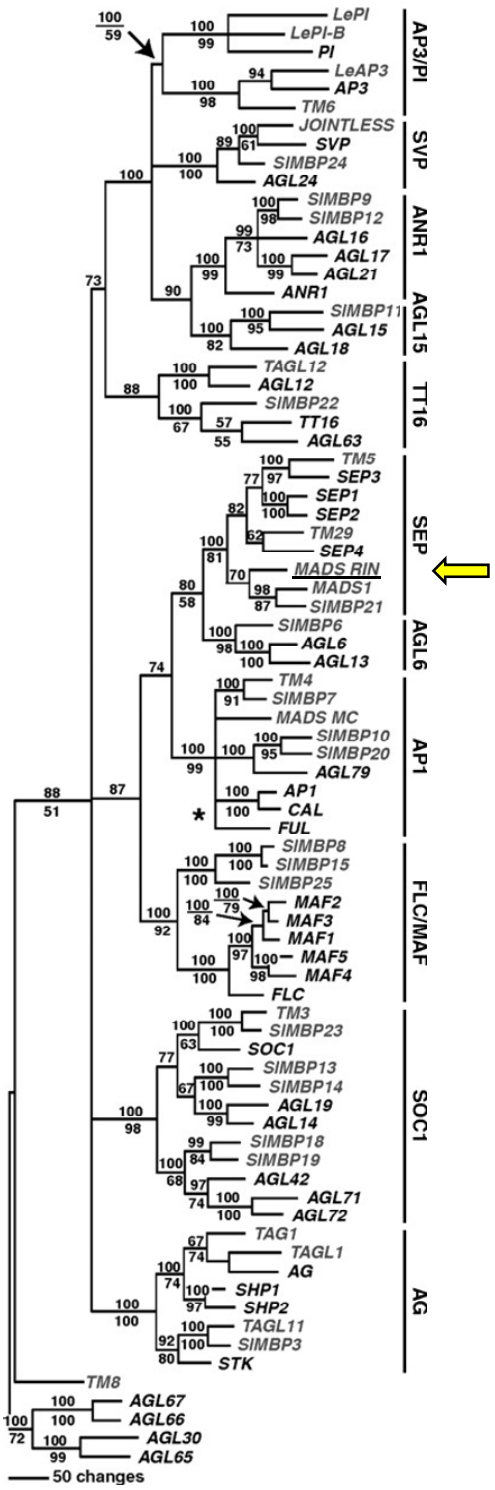


Figure 1-5 Phylogeny of *A. thaliana* and *S.lycopersicum* MADS box genes (modified from Hileman et al 2006). Yellow arrow indicates *RIN*.

An additional partner has been shown to be required for the proper activity of the ABC genes. The essential role of the four *SEPALLATA* (*SEP*) genes (*SEP1, 2, 3, 4*) in floral organ identity is illustrated by the quadruple *sep* mutant which develops flowers containing only leaf like organs (Robles and Pelaz, 2005). The observation that the expression of ABC class genes is not affected in the quadruple *sep* mutant indicates that the SEP proteins do not control the expression of ABC genes, but rather the manifestation of their activities (Angenent et al., 1994; Pnueli et al., 1994; Kotilainen et al., 2000). Further studies have shown a direct protein-protein interaction between SEP and ABC proteins (Fan et al., 1997; Egea-Cortines et al., 1999; Honma and Goto, 2001; Pelaz et al., 2001). The updated floral organ identity model has been modified to include the *SEP* genes, now referred to as the E class of floral development genes. In this model, the SEP proteins are required for the formation of a transcriptionally functional tetrameric complex involving the ABC proteins (Honma and Goto, 2001). This model is supported by the observation that ectopic expression of ABC and E genes is sufficient to convert leaves into floral organs, whereas the expression of ABC genes alone (i.e. absent E function genes) fails to do so (Honma and Goto, 2001; Pelaz et al., 2001). The close phylogenic relationship between *RIN* and *SEP* genes suggest that the RIN protein might also be involved in the formation of higher order complexes in ripening fruits.

1.3.2 NAC domain transcription factors

1.3.2.1 *Description*

The *NOR* gene, located on tomato chromosome 10, belongs to the NAC family; the largest plant-specific family of transcription factors with more than 100 members identified in *A. thaliana* (Riechmann et al., 2000). *NAC* genes are named for their

founding members: *P. hybrida NAM* and *A. thaliana ATAF1/2* and *CUC2* genes (Souer et al., 1996; Aida et al., 1997). Mutations in the *NAM* and *CUC2* genes produce seedlings with fused cotyledons lacking a shoot apical meristem (SAM) (Souer et al., 1996; Aida et al., 1997). Although generally seedling lethal, rescued shoots from these mutants also develop abnormal flowers with fused sepals and stamens (Aida et al., 1997). In conjunction with the fused cotyledon phenotype, this floral phenotype reveals the important role these genes play in defining organ boundaries. A *S. lycopersicum* homolog of the *CUC2* gene, *GOBLET* (*GOB*), has recently been characterized and shown to regulate the formation of tomato compound leaf structure, shoot apical meristem (SAM) formation and organ separation (Berger et al., 2009). Following the cloning and characterization of *NAM* and *CUC2*, numerous other genes belonging to the NAC family of transcription factors have been described in many plant species including those belonging to monocots, dicots, conifers and mosses (Olsen et al., 2005). These genes are involved in numerous processes, including the regulation of several developmental programs (SAM formation, definition of organ boundaries, lateral root formation, flower development, senescence) (Souer et al., 1996; Aida et al., 1997; Sablowski and Meyerowitz, 1998; Takada et al., 2001; Hegedus et al., 2003; Vroemen et al., 2003; Weir et al., 2004), biotic and abiotic stress responses (Xie et al., 1999; Ren et al., 2000; Collinge and Boller, 2001; Hegedus et al., 2003; Fujita et al., 2004; Tran et al., 2004), hormone responses (Seki et al., 2002; Furutani et al., 2004), light response, and secondary cell wall formation (Mitsuda et al., 2005; Zhao et al., 2005; Zhong et al., 2006; Ko et al., 2007; Mitsuda et al., 2007; Zhong et al., 2007; Mitsuda and Ohme-Takagi, 2008; Yamaguchi et al., 2008; Yamaguchi et al., 2010; Yamaguchi et al., 2010; Zhao et al., 2010).

1.3.2.2 *Structure of NAC proteins*

The NAC transcription factors are characterized by a conserved N-terminal domain, the NAC domain, and a more variable C-terminal domain (CTD) (Figure 1-6).

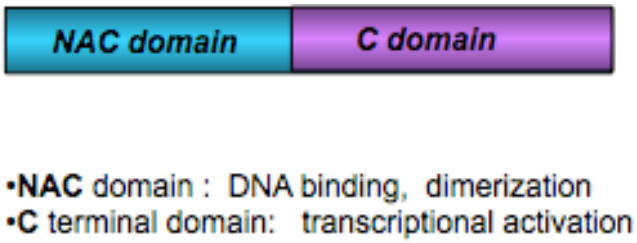


Figure1-6 Structure of plant NAC protein.

The NAC domain possesses a DNA-binding motif composed of a twisted anti-parallel β-sheet flanked on each side by two α-helices (Ernst et al., 2004; Olsen et al., 2004). NAC proteins have been showed to homo- and heterodimerize through the N-terminal region of the NAC domain (Xie et al., 1999; Hegedus et al., 2003; Olsen et al., 2004). The CTD region of *NAC* genes, though highly variable between different family members, often displays transcriptional activity (Xie et al., 1999; Ren et al., 2000; Duval et al., 2002; Hegedus et al., 2003; Fujita et al., 2004; Robertson, 2004; Tran et al., 2004).

Several studies have examined the DNA binding activity of NAC proteins (Ernst et al., 2004; Tran et al., 2004; Olsen et al., 2005). *In vitro* enrichment assays using the Arabidopsis ANAC019 and ANAC092 proteins identified a NAC binding site (NACBS) consensus sequence (CGT(G/A)) as the preferred core binding motif of NACs (Olsen et al., 2005). *In vitro* gel retardation experiments further indicated that NAC proteins have a stronger affinity for DNA regions composed of two palindromically arranged NACBS sequences. This preference and the observation that

removal of the dimerization domain of the ANAC protein abolishes binding to NACBS motifs indicates that dimerization is required for DNA binding activity (Olsen et al., 2005). Interestingly, examination of promoter sequences of putative *ANAC019* target genes identified by microarray analysis (Fujita et al., 2004; Tran et al., 2004) indicate that most lack the expected palindromic copies of NACBS. Many do however possess a single NACBS, suggesting that binding of a NAC protein to target *cis*-elements can be mediated by single NACBS *in vivo*. Interestingly, whereas MADS box protein functional specificity is mainly determined by the C-terminal IKC region, the functional specificity of NAC proteins is determined by their DNA binding NAC domain. This conclusion was based on experiments showing that substitution of the C-terminal region of NACs with unrelated transcriptional activation domain (e.g. VP16) has been shown to be sufficient to mimic normal protein activity (Taoka et al., 2004).

1.3.2.3 *NAC Phylogeny*

Phylogenetic analysis using sequences of the NAC domain of the 105 and 75 NAC proteins found in *A. thaliana* and *O. sativa*, respectively, distinguished two major groups of NAC proteins, designated as group I and II (Ooka et al., 2003). Those two groups can further be subdivided into 18 subgroups based on the sequence of the CTD domain (Ooka et al., 2003). Analysis of sequences retrieved from the Tomato Transcription Factor Database (Yang et al., 2010) (planttgdb.cbi.pku.ed.cn) and several functional studies (Selth et al., 2005; Berger et al., 2009; Yang et al., 2010) indicate that the tomato genome encodes at least 44 NAC proteins (Yang et al., 2010). This number is likely an underestimation of the actual number of NAC genes in tomato, since it was based on incomplete gene sequences.

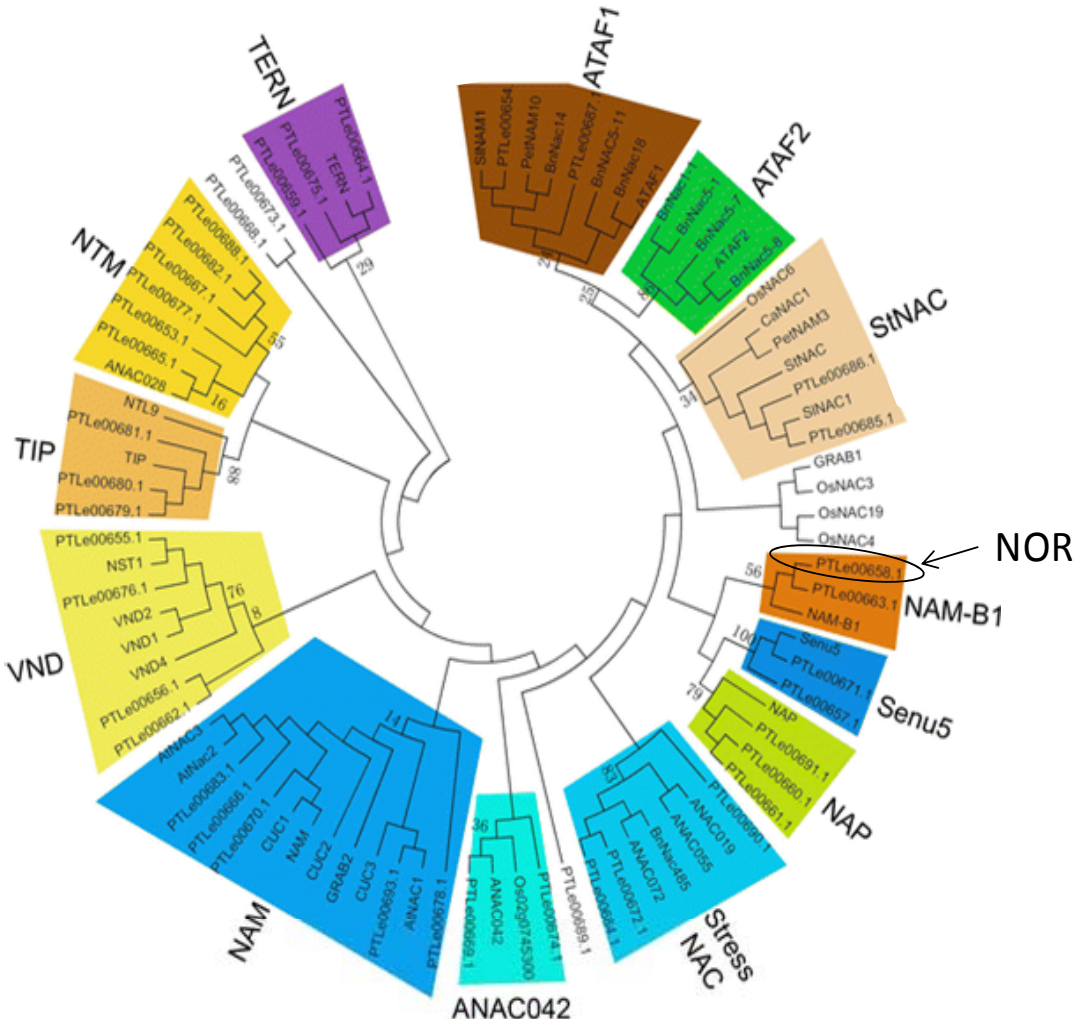


Figure 1-7 Phylogeny NAC genes in tomato and other species. NOR gene (PTLe00658.1) identified by black arrow. From Yang et al. (2010)

Yang et al. (2010) published a phylogenetic tree describing the relationship between 43 tomato NAC proteins and 42 NACs from other plant species. This analysis revealed that NOR belongs to the NAM-B1 subfamily, forming a monophyletic group with the wheat senescence protein NAM B1 (Uauy et al., 2006) and another NAC protein from tomato (PTLe00663.1, Fig 1.7). The phylogenetic tree also reveals that the NAM-B1 subgroup is closely related to the NAP subgroup. The *A.thaliana NAP* gene was shown to be directly regulated by the AP3/PI MADS box protein in developing flowers (Sablowski and Meyerowitz, 1998). Interestingly, the apple *PI* gene is necessary for normal fruit development as a natural mutation results in parthenocarpy (Yao et al., 2001). The tomato *PI* gene remains to be functionally characterized.

1.4 OBJECTIVE OF THE DISSERTATION RESEARCH

The molecular mechanisms controlling fleshy fruit ripening involve the coordination of multiple biochemical pathways and judging from the phenotype of their respective mutants, *RIN* and *NOR* play key roles in the regulation of these pathways. The fact that both genes encode transcription factors suggests that they act as master regulators of the ripening cascade and may be candidates for functional conservation of ripening control in other species. The objective of the work presented in the research described in this dissertation is to provide a better characterization of the specific roles of these transcription factors during tomato fruit ripening.

CHAPTER 2 IDENTIFICATION OF RIN PRIMARY TARGETS DURING TOMATO RIPENING

2.1 INTRODUCTION

Ripening is a complex developmental process responsible for the transformation of the seed-bearing structure of fleshy fruit species into a palatable and nutritious tissue attractive to seed dispersing organisms and consumers (Seymour, 1993). Although the exact physiological and chemical changes associated with fruit ripening differ among fruit from different species, some key general events are characteristic of most. These include modification of the cell wall structure, starch hydrolysis, changes in composition and levels of secondary metabolites, and increased susceptibility to pathogens (Seymour, 1993). These phenotypic changes are the result of the coordinated activation of many different molecular pathways, the genetic regulation of which has been a subject of research for more than 30 years (Seymour, 1993; Giovannoni, 2007)

Fleshy fruits have traditionally been divided into two categories based on their ripening behavior. Climacteric fruit are characterized by a burst of respiration typically associated with a dramatic increase in ethylene production at the onset of ripening, whereas non-climacteric fruits do not (Lelievre, 1997). Numerous studies have demonstrated that ethylene is essential for the ripening of climacteric fruits (Seymour, 1993). This requirement is demonstrated by that fact that climacteric fruit that are defective in ethylene production, perception or signaling fail to ripen properly. Furthermore, the exogenous application of ethylene blocking agents, such as 1-methyl cyclopropene (1-MCP) or silver, efficiently prevents ripening of climacteric fruits (Saltveit and Dilley, 1978; Hobson, 1984; Dupille, 1995).

The ethylene signalling pathway is well conserved between *A. thaliana* and *S. lycopersicum*, the latter being an important model species for climacteric fruit. Studies of both organisms have led to a general understanding of ethylene's mechanism of action (Wilkinson et al., 1997; Bleecker and Kende, 2000; Stepanova and Ecker, 2000). Briefly, ethylene is synthesized from the methionine-derived compound *S*-adenosyl-methionine (SAM) through the sequential action of ACC synthase (ACS) and ACC oxidase (ACO) enzymes. Ethylene perception is mediated by ER–bound receptors, termed ETR (*ethylene response*) which resemble bacterial two-component histidine kinases. ETRs are negative regulators of ethylene signalling as dominant mutations leading to ethylene insensitivity have been shown to result from constitutive activity of mutant receptors (Chang et al., 1993). Receptor activity is blocked upon binding of ethylene, leading to the activation of a MAPK signalling cascade culminating with the activation of the transcription factor EIN3(Bleecker and Kende, 2000; Alexander and Grierson, 2002). EIN3 is in turn responsible for the regulation of numerous other ethylene responsive genes (Solano et al., 1998) including the secondary transcription factors known as *ethylene response factors* (ERFs). Numerous homologues of each of these ethylene components have been identified in tomato, some of which are expressed in a fruit specific manner (Rottmann et al., 1991; Barry et al., 2000).

An important feature of the ethylene response occurring during climacteric fruit ripening is the switch from an auto-inhibitory (system1) to an autocatalytic (system 2) response (Barry et al., 2000; Barry, 2007). More specifically, ethylene production in vegetative tissues and immature fruits is controlled by a negative feedback loop that results in reduction of ACS and ACO activities following exposure to ethylene (Alexander and Grierson, 2002). In contrast, in ripening fruits and in some senescing flowers, ethylene perception triggers an autocatalytic positive feedback loop resulting

in a dramatic increase in the rate of ethylene synthesis. This transition from system 1 to system 2 is necessary for the large ethylene production associated with the initiation of the ripening program of climacteric fruits (Barry, 2007). The exact mechanism by which this change in sensitivity is achieved at the onset of ripening remains unclear and is a primary interest of our laboratory.

The transition from system 1 to system 2 highlights the existence of a developmentally regulated signal acting before the ethylene burst and responsible for the acquisition of a competency to ripen (Lelievre, 1997). The existence of a state of ripening competency is demonstrated by several tomato mutants, including *ripening inhibitor (rin)* (Vrebalov et al., 2002) and *non-ripening (nor)* (Giovannoni, 2004). The fruits of these mutants are unable to ripen and lack most of the ripening phenotypes, remaining green and firm and failing to produce the typical burst in ethylene. Lack of ethylene production is however not the cause of their lack of ripening since exogenous application of ethylene fails to rescue the non-ripening phenotype(Giovannoni, 2007). The observation that some ethylene-dependent transcription is observed after exogenous ethylene application indicates that ethylene perception and signalling is largely functional in these mutants (Lincoln and Fischer, 1988). *rin* and *nor* mutations therefore specifically affect the ability of fruit to correctly respond to ethylene and the corresponding protein are likely to be involved in ripening competency acquisition. The cloning of the *RIN* gene *(*Vrebalov et al., 2002) has shed some light on the upstream events leading to ripening competency. RIN is a member of the MADS box family of transcription regulators, known to play essential roles in a variety of developmental processes such as control of vegetative growth, flowering time, as well as formation of flower and reproductive structures (Ng and Yanofsky, 2001). The dramatic phenotypic effect of the *RIN* mutation suggests its role as a master regulator of the ripening cascade.

The precise regulation and coordination of several biochemical pathways underlies the complex phenotypic changes occurring during fruit ripening. Howvever, the exact mechanism by which RIN regulates the expression of the numerous downstream genes involved in the different aspects of fruit ripening remains elusive. RIN could directly control the transcription of effector genes responsible for each ripening phenotype, or could regulate a second layer of pathway-specific transcription factors required for the control of these effectors genes. A detailed characterization of RIN activity represents an essential step toward the elucidation of the developmental transcriptional network leading to ripening.

In order to gain a better understanding of the regulatory network underlying ripening competency acquisition, I employed a chromatin immunoprecipitation strategy to identify the primary targets of RIN. In summary, I showed that RIN interacts with promoters of many genes reflecting all major ripening pathways. As such, I provide evidence that RIN is a master regulator of ripening that directly influences many ripening-associated genes.

2.2 METHODS

Plants

Wt and *rin* (*rin/rin*) mutant tomato plant (cv Ailsa Craig) were grown under normal greenhouse condition until maturity. Fruits were staged based on the number of days from anthesis to breaker stage as defined by the detection of orange color at the base of wt fruits.

Constructs

The construct pET-RIN-KC was obtained by PCR amplification of pET-RIN using primers RIN KC-F (5'-TATA***GGTACC***GGTGAGGATTTGGGACAATTG-3') and pET28a R (5'-TATA***GGTACC***CATTTGCTGTCCACCAGTC-3') digesting with

KpnI and *DpnI* and ligating. pET-RIN was obtain by PCR, amplifying full length RIN cDNA using primers RIN F (5'-TTTTGGATCC***GAATTC***ATGGGTAGAGGGA-AAGTAG-3') and RIN R (5'-TTTT***CTCGAG***TCAAAGCATCCATCCAGGTA-CAAC-3'), digesting with *EcoRI* and *XhoI* restriction enzymes and cloning into pET28a vector (Novagen) previously digested with the same enzymes.

Recombinant protein purification and antibody production

E.coli BL21 Star (DE3) (Invitrogen) cells containing pETRIN-KC were grown in LB at 30°C overnight. The next day a fresh Luria Broth (LB) culture was inoculated with the overnight culture diluted to 0.1 OD_{600} and grown for 3h at 30°C. IPTG was then added to a final concentration of 1mM and the culture incubated for another 3h at 30°C. The cells were pelleted by centrifugation and resuspended in TALON Equilibration buffer 1X pH8 (Clontech) containing 2mg/ml of lysozyme. Cells were lysed by sonication on ice using a Branson 450 sonicator (settings: power 4.5, duty 50%). The lysate was centrifuged at 14000g for 10min at 4°C and the supernatant discarded. The pellet was solubilized in TALON Equilibration buffer 1X pH8 containing 6M guanidine. Purification of the HIS-tagged RIN-KC protein was performed according to the TALON His Batch/Gravity-Flow Column purification protocol (Clontech). 1mg of purified His-RIN-KC protein, quantified using the BCA assay (Pierce), was sent to Covance Research Products (Denver,PA) for injection into rabbits to raise antibodies.

Protein extraction from fruit

Tomato (cv. Ailsa Craig) wt and *rin* fruits at different stages of development were frozen and ground in liquid nitrogen. Proteins were isolated from ground tissue using the protocol described by (Wang et al., 2006). Protein was quantified using the BCA assay (Pierce).

Western Blot

25µg aliquots of protein extracts were separated in SDS-PAGE gels and transfered to a nitrocellulose membrane using standard procedures (Current Protocol in Molecular Biology). Immunoblottings analyses were performed using the rabbit polyclonal RIN antibody at a 1:1,000 dilution and 1:100,000 anti-rabbit HRP secondary antibody (Sigma).

Chromatin Immunoprecipitation

Chromatin immunoprecipitaton was adapted from Fiil et al (CSH protocol, 2008), Manzara et al. (Manzara et al., 1991) and Nelson et al. (2006). For chromatin cross-linking, tomato fruit pericarp tissue was diced and place in a 50ml Falcon tube filled with MC buffer (10mM $KHPO_4$ pH7, 50mM NaCl, 0.1M sucrose, 1% formaldehyde). Vacuum was applied for 10min at a time for four times (5 min rest between vacuum applications) and one additional time in presence of 0.125M glycine to stop the crosslinking. Tissue was then rinsed with water, frozen in liquid nitrogen and ground with mortar and pestle. For each chromatin immunoprecipitation, 3g of the crosslinked tissue was resuspended for 30min at 4°C in 45ml of buffer 1 (0.4M sucrose, 10mM Tris pH8, 5mM β-mercapto-ethanol (BME), 1X plant protease inhibitors (Sigma)), filtered through 2 layers of Miracloth (Calbiochem) and centrifuged for 20min at 3000g. Pellets were resupsended in 1ml of buffer 2 (0.25M sucrose, 10mM Tris pH8, 10mM $MgCl_2$, 1% Triton X-100, 5mM BME, 1X plant protease inhibitor (Sigma)), transferred to a 1ml eppendorf and centrifuged for 10min at 12000g. Pellets were resuspended in 300µl of buffer 3 (1.7M sucrose, 10mM Tris pH8, 2mM $MgCl_2$, 0.15% Triton X-100, 5mM BME, 1X plant protease inhibitors), carefully layered on top of 1.5ml of buffer 3 (in a 2ml tube) and centrifuged for 60min at 16 000g. The resulting pellet was resuspended in 1ml of freezing buffer (100mM NaCl, 50mM HEPES pH7.6, 25%glycerol, 1mM EDTA, 5mM BME, protease inhibitor), frozen in liquid nitrogen and thawed on ice. 250µl of lysing buffer (2.5M

NaCl, 50mM HEPES pH 7.6, 1mM EDTA, 5mM BME) was added to the resupsended pellet and incubated for 30min at 4°C. The solution was then sonicated on ice (Branson Sonifier 450) for 10sec at a time (duty 15%, power 3) for a total of 40 sec (30sec on ice between sonication), centrifuged 10min at 16 000g and the chromatin containing supernatant was transferred to a 2ml tube. Supernatant was diluted 2 fold with 50mM Tris-Cl pH8, 1mM EDTA, 0.1% Triton X-100 and incubated for 1h at 4°C on a rotating wheel with 25ul of preimmune serum and 40µl of blocked protein A sepharose beads (GE Healthcare), blocked with 10ug/ml of BSA and 10ug/ml of salmon sperm DNA. The mixture was centrifuged for 10min at 16000g and the supernatant separate in three tubes for INPUT, preimmune and RIN ChIP treatments. Chromatin immunoprecipitation was performed on the preimmune and RIN ChIP samples by incubation with 25ul of blocked proteinA sepharose and 3µl of rabbit preimmune serum or RIN antibody serum, respectively, for 16h at 4°C on a rotating wheel. Samples were centrifuged 2 min at 2000g and the supernatant discarded. The sepharose beads were then washed 5 times with washing buffer (150mM NaCl, 50mM Tris pH8, 1mM EDTA, 1% Triton X-100) by rotation for 10min at room temperature followed by 2min centrifugation at 2000g. After the last wash was removed, 100µl of 10% Chelex resin solution (BioRad) was added to the beads and the INPUT sample and boiled for 10min. After cooling at room temperature, samples were incubated for 45min at 55°C with 20µg/ul of proteinase K. Samples were then boiled for 10min and centrifuged for 10min at 16000g (4°C) and the supernatant recovered. An additional 100µl of water was added to each tube, vortexed, centrifuged and the supernatant was then pooled with the previous supernatant solution. Samples were further purified using a Qiagen PCR purification column (Qiagen) following the manufacturer's instructions, except that the final elution was 100µl.

qPCR

Quantitative PCR was performed using SYBR green on a AB 7900 real-time PCR platform following the manufacturer instructions. Briefly, 2µl of gDNA sample obtained as described above was mixed with 5µl of SYBR Green and 300nM of promote- specific primer. Relative fold enrichements are calculated by dividing the amount of gene specific amplification by 18S amplification. Primers used in the qPCR reaction are listed in Table 2-1.

qRT-PCR

RNA was extracted from fruit tissue at different stages of development (days after anthesis) using the Plant RNA kit (Invitrogen) and following the manufacturer's instructions. RNA was then digested with RQ1 DNase (Promega), and further purified on Qiagen RNA purification columns. Quantitative RT-PCR was performed using SYBR Green on the AB7900 using 18S as the internal control. Gene specific primers used in the qRT-PCR assay are listed in Table 2-1.

Table 2-1 Primers used for gene expression (qRT-PCR) and Chromatin Immunoprecipitation (qPCR) enrichment measurements

Gene	Acc. no	Primer sequences(5'-3')	
		GENE EXPRESSION	CHIP
PG2a	X04583 X80908	F: TCAAGGGCACAAGTGCAACAAAGG R: TGCACGTAGCCTCTGATGGTTT	F GGATGCAGGGTTGTACAAAGAGAG R AGTCGTCGTATGAAGGCGAAAGGA
ACS2	X59139	F : A A G C G C G A T G A G G T T A G G T A R: AAAGTGGACGCAAATCCATC	F: TCATACGATCACAAACGAGCTATTCT R: GAAGATTGTTTGTACTATGTGGGAG
ACS4	M88487	F : AAATCTCCACCTTCACTAACGAAC R : CCTAAGTCCTTGGAAAGACTAGACAC	F: TCCAACCATACGTGCTATGCCCAA R: GCTTGTGCCAGACAAGAGATGT
RIN	AF448522	F : CATGGCATTGTGGTGAGCAAAGTGT R : AGCATCATGTGTTGATGGTGCTGC	F : CCATCCTGTTAGTGATATTGTCTGC R : TGAAGTGTACTGACATTCCGGT
NOR	AY573802.1	F : AGAGAACGATGCATGGAGGTTTGT R : ACTGGCTCAGGAAATTGGCAATGG	F: GCACCACCAATGGATGTGGTTCTT R: GTAGGCTTATTCGAATCTCTTCGC
E4	S44898	F : G A C C A C T C T A A A T C G C C A G G R: TTCCTGAGCGGTATTGCTTT	F: ACATCTTCACCCATGCAATCCA R: TGCTACTGCTGTTGTTACCCTCCA
E8	DQ317599 X13437	F : T G G C T C C G A A T C C T C C C A G T C T R: GTCCGCCTCTGCCACTGAGC	F: CTGCACAAGACTTTCTATTGTTGGG R: ACGTCTAGAAAGACCCATATCT
CNR	DQ672601	F : C G G C A A C T C C T C T T A G C A T C R: GCCACAAGGTGTGTGAGTTC	F : TCCACCTTTCCTGGCCCACT R: ACGTGTGAGGACCACCAGTTCCA
HB1	AC215430	F : C A A T C G G A G G A A G A T G A T G G R: TGTTCATGGTGCTGCTCTTC	F: CACCAAGGGAAACGGAAAGGAGTT R: TGCCTTGGTCTCCTCATATTCCCA
ACO1	X58273	F : T G G A G A T G A G A G A G C C A A C A R: TTCCATGGTTCACCAACTCA	F: GGCATGTTCCTTCTAACTTAATTAGCATTC R: TGAGAGGTTCACAAATTCTCCCTC
NR	AY600437	F : A T C A G G T T G C T G T C G C T C T T R: GGCCATCTCTGCTTCTTGTC	F: GGGTACTATGTCATGTTCCGTCAC R: TCTAGTGTCTTCCACTAACCTCCC
EXP	AF443209	F: TGGTTCCTTCTCATTGGCAATTTGG R: TTCAGTGAGGACTCGATTTCTTTTCC	F : CAAATTTAGTTAAATAGTGGGAGCGGA R: AGGTTTCCCATGGAACACTTGCAG
PDS	X78271	F: ACTGTTATTTTTCAGTAAAATGCCTCA R: ACCTCGAGCTCCAAAGATAAGCT	F: TGTTGAGCACTTTGTGTGCATTGG R: TTTGTCCTTCACGAGGACTCGGTT
TDR4	X60757.1	F : A C T G G A C T C T C C T C A C C T T G G G G R: AGCTGCACCTTGCTGCTGTGA	F: TTCCATTGTTTCGCATCACCTGGC R: CCAGAGTGGTTTCGTCAAATGTGT
PSY1	EF157835	F : A C A G G C A G G T C T A T C C G A T G R: AGCTCAATTCTGTCACGCCT	F: ACGAGGAGTAAGGTTTGCAACGAC R: TGACTTGTCCAATAATTTAGGGCG

2.3 RESULTS

2.3.1 Transcriptomic analysis of *rin* mutant fruit

The *rin* mutant produces fruits that fail to ripen, even in presence of exogenous ethylene application (Vrebalov et al., 2002). In order to gain a better understanding of the global effect of the *rin* mutation on gene expression at the onset of ripening, RNA extracted from wild type (wt) cv. Ailsa Craig (Ac) and *rin* fruits at the breaker stage (defined as 41dpa) was compared to RNA extracted from Ac at the MG stage using the TOM1 microarray platform (Alba et al., 2004). A total of 547 features (spots) corresponding to 349 non redundant unigenes were found to be significantly ($p<0.05$) differentially expressed (> 2 fold) at the BK stage between *rin* and Ac fruits. The heat map of Figure 2-1A illustrates the relative level of expression of these features in *rin* and Ac BK fruit when compared with Ac MG fruit. Figure 2-1B shows the functional distribution of the identified unigenes for which a Gene Ontology (GO) term is available. Among the genes that show significant up regulation in the *rin* fruit compared to Ac, a large proportion is associated with photosynthetic activity. This observation is consistent with the stay-green phenotype of the *rin* fruit and suggests that these fruits fail to efficiently reduce the expression of chloroplast associated genes; a phenomenon normally associated with the transition of chloroplasts to chromoplasts during ripening. Several other categories of genes show a decrease in expression in *rin* fruits, including genes involved in primary and secondary metabolism, signal transduction and hormone responses. Fruit ripening typically involves important changes in all these categories; consequently down-regulation of associated genes suggests that the protein encoded by the *RIN* gene has a large effect on ripening. Interestingly, genes belonging to the transcription factor category are highly represented in both up and down regulated genes.

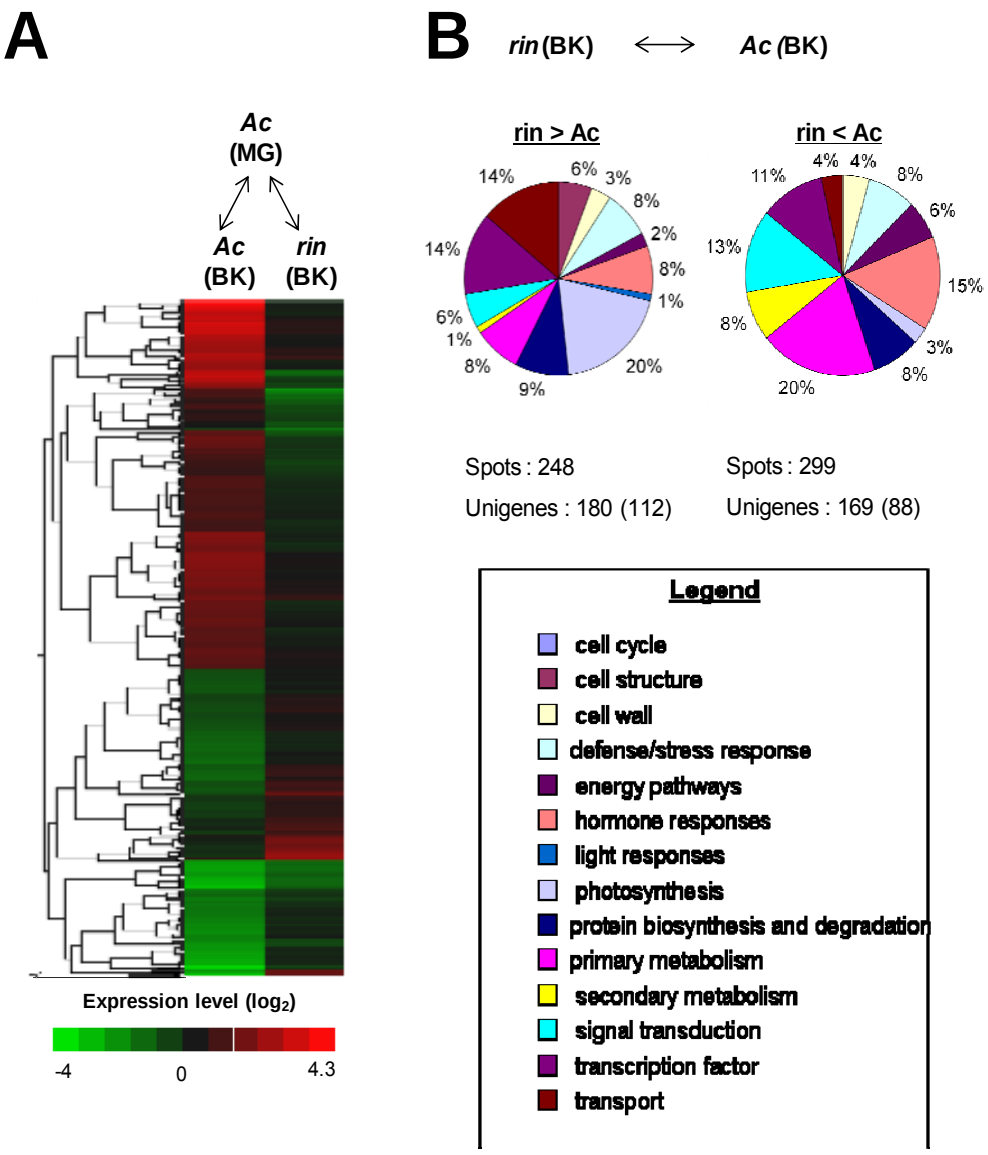


Figure 2-1 rin fruit transcriptome profiling. A) Heat map showing expression level of TOM1 microarray features in Ac and *rin* fruits compared to Ac MG fruits. Only genes showing significant ($p<0.05$) differential expression (>2fold) between BK stage Ac and *rin* fruit are depicted. B) Distribution of up and down regulated genes between *rin* and Ac BK fruits based on their gene ontology (GO).

This supports the hypothesis that RIN could act as an important regulatory switch in the transition to fruit ripening, positively and negatively regulating numerous secondary transcription factors. In that regard, RIN can be seen as a master regulator of fruit ripening. However, the molecular underlying mechanistic details remain unclear. The present study focuses on the characterization of RIN mechanism of action.

2.3.2 Production of a RIN-specific antibody

In order to study the endogenous function and regulation of the RIN protein, I developed a polyclonal antibody that would specifically detect it. RIN shares highly a conserved N-terminal DNA binding domain with other members of the MADS box family, whereas the C-terminal portion, whose functions include protein-protein interactions and transcriptional activation, is more variable (Kaufmann et al., 2005). In order to obtain an antibody that would specifically recognize RIN and not other members of the family, I raised the antibody against the C-terminal (more variable portion) of RIN. His-tagged recombinant protein encoding the RIN C-terminal portion was produced in *E. coli* and purified on a His-binding column. The purified protein was used to raise polyclonal antibodies from rabbit. In order to test the specificity of the antibody, a western blot of tomato fruit proteins at the Breaker stage (BK, 41dpa) was performed. As seen in Figure 2-2A the RIN antibody recognizes a 28kD band in the wt fruit extract (Ac). This size corresponds to the expected molecular weight of the RIN protein. The fact that this band is not detected in *rin* mutant protein extract but is detected in *rin* complementation lines (35S::*RIN* in *rin*) further supports the conclusion that this band correspond to the RIN protein. A higher molecular weight band (48kDa) is detected in the *rin* mutant protein extract.

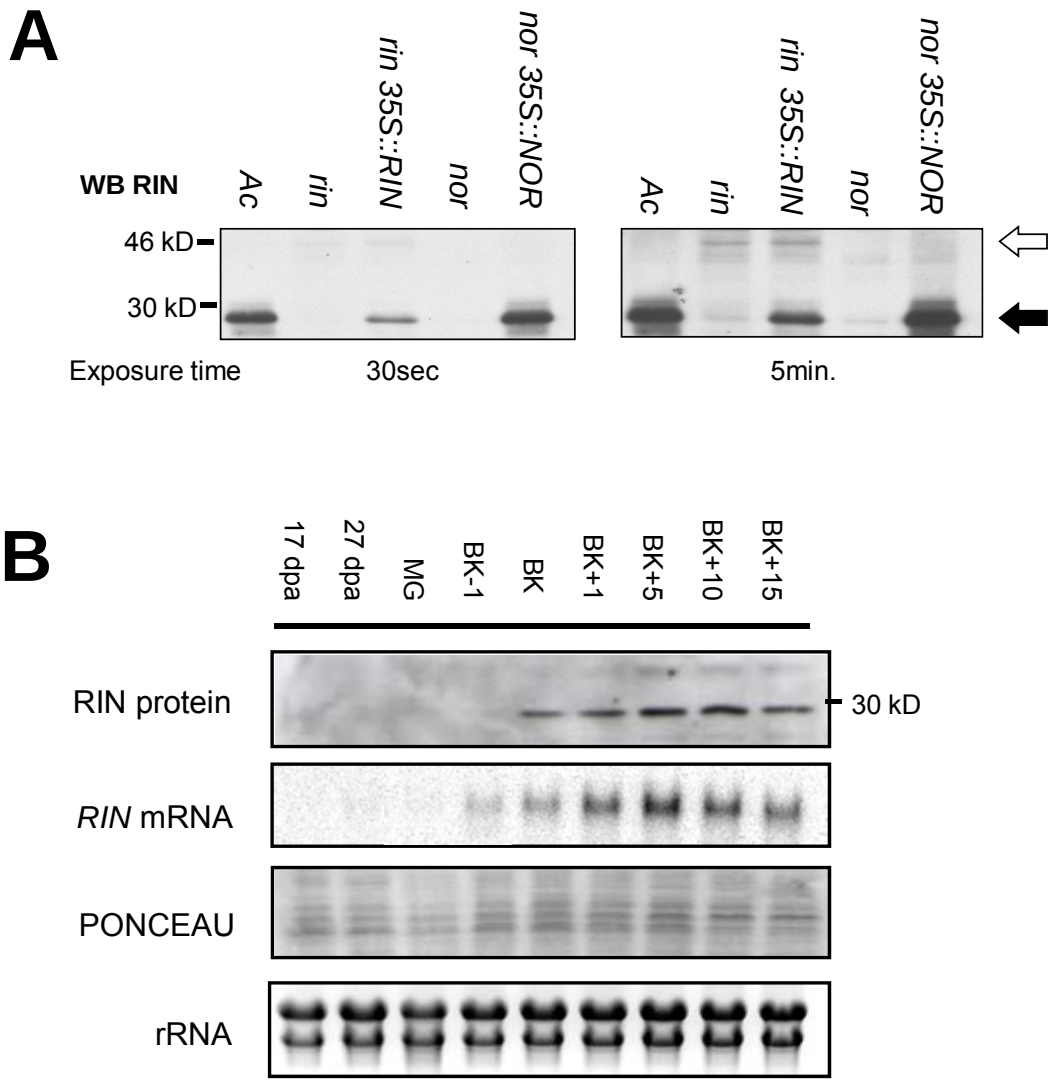


Figure 2-2 RIN specific antibody. A) Western blot of Breaker + 2 days-staged fruit from different tomato genotypes using RIN antibody. Black arrow: RIN protein (28kDa), white arrow: RIN-MC chimera protein (45kDa) . B) Time course of RIN protein and mRNA accumulation during fruit development and ripening, as revealed by Western blot and Northern blot, respectively. Dpa = day post anthesis; MG= mature green stage, BK = breaker stage, BK+X = breaker +X day. Ponceau S staining and rRNA are used as loading control for protein and mRNA gels, respectively.

This band likely corresponds to the chimeric RIN-MC protein produced in this mutant (Vrebalov et al., 2002). Ito et al. (2008) have also published data about a RIN antibody; however the reported protein their antibody detects is apparently of a higher molecular weight (35kDa) than the expected molecular mass of the RIN protein. Having confirmed the specificity of the antibody, I next examined the behavior of RIN protein during fruit development and ripening using Western and Northern blot analyses. Figure 2-2B shows RIN protein accumulation at different stages of fruit development. RIN is absent during the initial phase of fruit developmental (0 to 35 dpa), and starts to accumulate early during ripening. RIN is detected slightly before the BK stage and its expression is maintained throughout ripening (up to 15 day after breaker). Comparison of protein and RNA expression profiles reveal a tight correlation between the two, suggesting that the accumulation of the *RIN* protein is mainly regulated at the level of gene transcription (Figure 2-2B).

2.3.3 Identification of promoters that associate with RIN

RIN is proposed to act as master regulator of ripening by controlling the expression of numerous genes. Although its role in influencing ripening-associated traits, such as cell wall degradation and ethylene production, is clearly supported by the strong phenotype of the *rin* mutant, the mechanism by which it exerts its effect remains unclear. In order to better understand the mechanism by which RIN influences ripening, a Chromatin Immunoprecipitation (ChIP) strategy was deployed (Wang et al., 2002). To perform this assay, tomato fruits were harvested at BK stage and cross-linked using formaldehyde. RIN or preimmune sera were then used to isolate protein-bound chromatin fragments. Quantitative PCR (qPCR) was used to measure an enrichment ratio following immunoprecipitation. Enrichment is defined here as the

amount of gene-specific DNA relative to the amount of control 18S gDNA (unbound control). Association of RIN to a particular DNA fragment should result in an increase in the enrichment level following RIN immunoprecipitation compared to the its level in the Input sample (prior to Immunoprecipitation) and preImmune (non-specific) IP. The specificity of the immunoprecipitation was further assessed by performing the ChIP on both *wt* and *rin* mutant tissues.

The ability of RIN to associate with four broad classes of ripening-associated genes which encompass many primary ripening responses was examined. Candidates included: (1) transcription factors, (2) ethylene pathway components, (3) cell wall metabolism genes and (4) carotenoid biosynthesis pathway genes. Since the tomato genome had not been fully sequenced at the time of the experiment, the choice of candidate genes in each of these categories was limited by promoter sequence availability.

2.3.3.1 Transcription factors

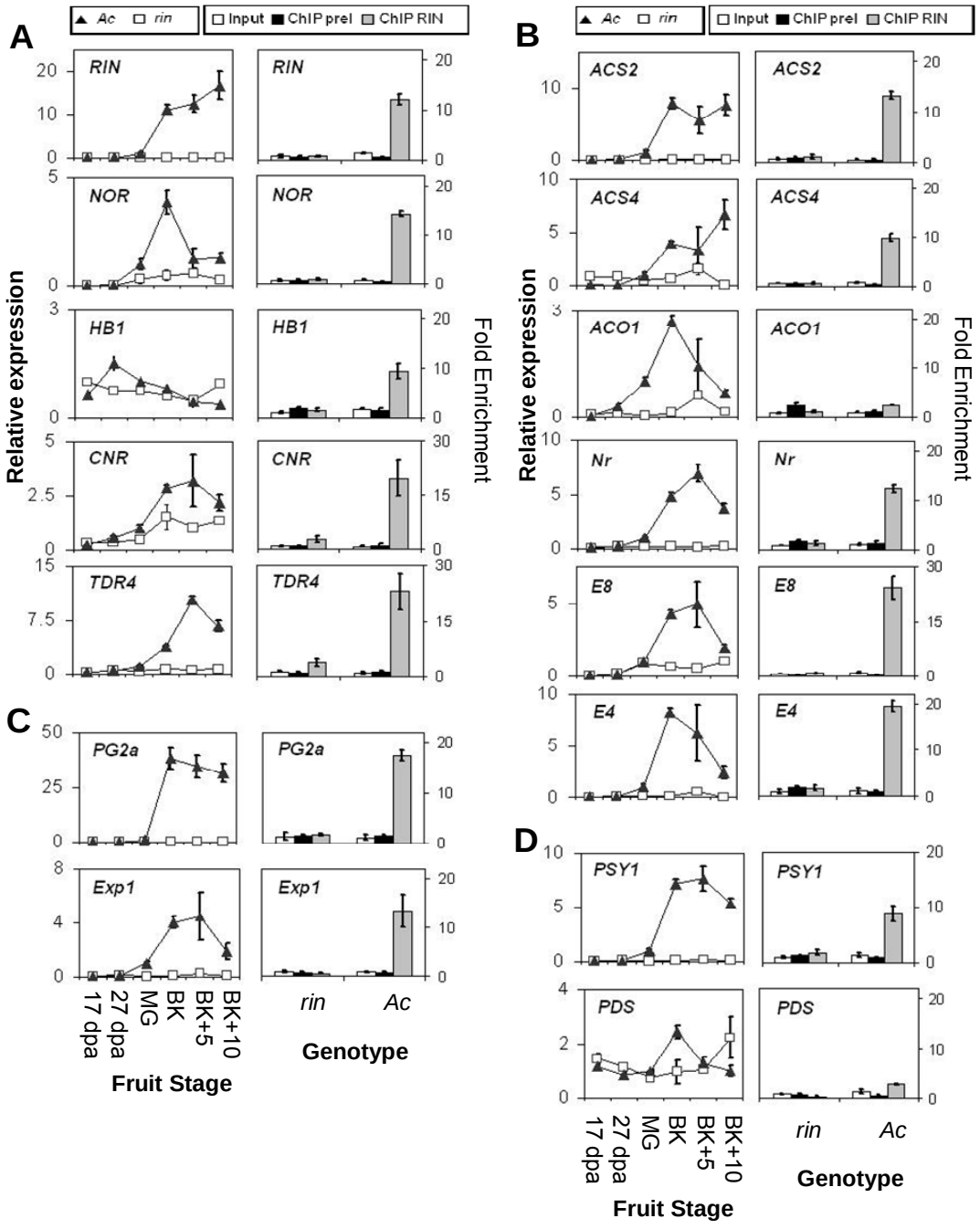
The first genes tested were the transcription factors. In addition to RIN, which is the focus of the current study, other transcription factors play important roles in fruit ripening. *NOR* is a NAC domain transcription factor whose mutation leads to a non-ripening phenotype similar to that observed in *rin* (Giovannoni, 2007). *LeHB-1* is an HD-Zip transcription factor that has been shown to positively control the expression of the ethylene producing enzyme ACO1 during fruit development and ripening (Lin et al., 2008). *CNR* is a SBP box transcription factor (Manning et al., 2006) a mutation in which leads to pleiotropic non-ripening phenotypes, including a mealy and pale pericarp (Fraser et al., 2001; Orfila et al., 2002; Eriksson et al., 2004). TDR4 is another member of the MADS box transcription family whose expression pattern suggests a possible role during fruit ripening (Busi et al., 2003).

In order to examine the expression level of these candidate genes during the different stages of fruit development and ripening, qRT-PCR was first performed. Figure 2-3A (left panel) shows the relative expression level of each gene in wt and similarly aged *rin* fruits. As expected, *RIN*, *NOR* and *CNR* transcripts increased dramatically at the onset of ripening in wt frui,t but these increases are not observed in the *rin* fruit, consistent with the hypothesis that RIN regulates their expression. The expression of LeHB1 remained constant during the early stage of fruit development and decreased during ripening, consistent with previously published data (Lin et al., 2008).
Using the ChIP-qPCR procedure described above I measured the ability of RIN protein to interact with the promoter region of each of these genes (Figure 2-3A-right panel). All promoters tested showed a clear enrichment following RIN immunoprecipitation in *wt* but not *rin* fruit, indicating an association with RIN.

2.3.3.2 *Ethylene components*

Since RIN is expressed prior to the onset of climacteric ethylene synthesis, it might be directly involved in regulating one or more components of the pathway. Previous studies have functionally elucidated the genes involved in ethylene synthesis, perception and signaling during tomato fruit ripening (Alexander and Grierson, 2002; Adams-Phillips et al., 2004). The increase in ethylene production is driven by the biosynthetic genes *ACS2*, *ACS4* and *ACO1*(Barry, 2007). While the ethylene receptor Never-ripe (*NR*/*LeETR3*) has been shown to play a major role during fruit ripening since a dominant mutation leads to insensitivity to ethylene and inhibition of ripening (Wilkinson et al., 1997). The *E4* and *E8* genes have initially been identified as rapidly induced in unripe fruit exposed to ethylene and during normal fruit ripening (Lincoln et al., 1987) .

Figure 2-3 Regulation of gene transcription by RIN. (**Left panel**) mRNA levels of transcription factor genes in Ac (wt) and *rin* mutant during tomato fruit development mesured by qRT-PCR. dpa = day post anthesis; MG=Mature Green stage; BK=Breaker stage. (**Right panel**) Chromatin immunoprecipitation of ripening-related promoters using RIN antibody. Enrichment corresponds to the ratio of gene specific amplification to g18S amplification measured by qPCR. BK staged *rin* and Ac fruits were used for the assay. Input= enrichment before immunoprecipitation; ChIP preI : enrichment following immunoprecipitation using preImmune serum; ChIP RIN = enrichment following immunoprecipitation using serum containing RIN antibodies. Gene categories : A) Transcription factor, B) Ethylene components, C) Cell wall modifying genes, D) Carotenoid biosynthesis genes.


A
Ac
rin
Input
ChIP prel
ChIP RIN
RIN
NOR
HB1
CNR
TDR4
Relative expression
Fold Enrichment
B
ACS2
ACS4
ACO1
Nr
E8
E4
C
PG2a
Exp1
D
PSY1
PDS
17 dpa
27 dpa
MG
BK
BK+5
BK+10
Fruit Stage
rin
Ac
Genotype

E8 encodes a protein similar to *E.coli* dioxygenase and has been shown to be a negative regulator of ethylene synthesis during ripening (Penarrubia et al., 1992; Kneissl and Deikman, 1996). *E4* belongs to the peptide methionine sulfoxide reductase family, but its role in the ripening remains uncertain. The *E4* and *E8* promoters have been extensively studied and dissected to understand the ethylene-dependant and independent *cis* elements regulating their expression during ripening (Deikman and Fischer, 1988; Cordes et al., 1989; Deikman et al., 1992; Penarrubia et al., 1992; Nicholass et al., 1995; Kneissl and Deikman, 1996; Xu et al., 1996; Deikman et al., 1998; Yokotani et al., 2004).

Figure 2-3B (left) shows the normal accumulation pattern of these genes in *wt* and *rin* fruits, and as expected, the *rin* mutation prevents the normal mRNA accumulation of each of these genes. Figure 2-3B (right) shows the result of the ChIP assay. The results show that RIN associate with the *ACS2* and *ACS4* promoters but not to the *ACO1* promoter. The promoter of the ethylene receptor *NR* also shows enrichment following RIN immunoprecipitation. The ChIP assay also reveals a very strong enrichment of the *E8* and *E4* promoters following RIN immunoprecipitation.

2.3.3.3 *Cell wall metabolism*

Two genes involved in ripening related cell wall modifications were tested for direct regulation by RIN: *Polygalacturonase2a* (*PG2a*) and *Expansin1*(*LeExp1*). Both genes are upregulated during normal ripening but not in the *rin* mutant (Figure 2-3C right). PG2a is an enzyme involved in pectin depolymerization and is highly up regulated during fruit ripening in tomato (Dellapenna et al., 1989). The promoter of the *PG2a* gene has been intensively studied and shown to possess both ethylene-dependent and independent *cis* elements (Montgomery et al., 1993; Nicholass et al., 1995). *LeExp* plays an important role in the fruit softening process that accompany

ripening although its exact molecular mechanism of action is not known (Rose et al., 2000). Our ChIP assay (Figure 2-3C-left) indicates that RIN associates with the promoter of the *PG2a* and *LeExp* genes in a developmentally specific manner.

2.3.3.4 *Carotenoid metabolism*

The tomato genes and corresponding enzymes involved in the biochemical transformation of isoprenoids to C40-long chain carotenoids have been characterized in previous studies (Cunningham and Gantt, 1998). Thus the promoter sequences for the phytoene synthase 1 (*PSY1*) and phytoene desaturase (*PDS*) genes were available and their interaction with RIN examined using the ChIP assay. PSY1 is the fruit specific isoenzyme responsible for the initiation of the carotenoid biosynthetic cascade during ripening, combining two molecules of GGDP (geranyl-geranyl diphosphate) into one molecule of phytoene (Bartley et al., 1992; Ray et al., 1992). PDS catalyses the second step of the pathway by converting phytoene into z-carotene, a precursor of lycopene (Pecker et al., 1992). The *PSY1* gene shows an increase in expression at the onset of ripening in wt but not in *rin* mutant fruits (Figure 2-3D-left). The expression pattern of PDS remains fairly constant throughout fruit development although a slight but reproducible increase occurs at the BK stage. This suggests that PDS is not a major limiting enzyme in carotenoid biosynthesis during fruit ripening. Interestingly, only the promoter of the *PSY1* gene was enriched following ChIP (Figure 2-3D-right), a result consistent with gene expression data.

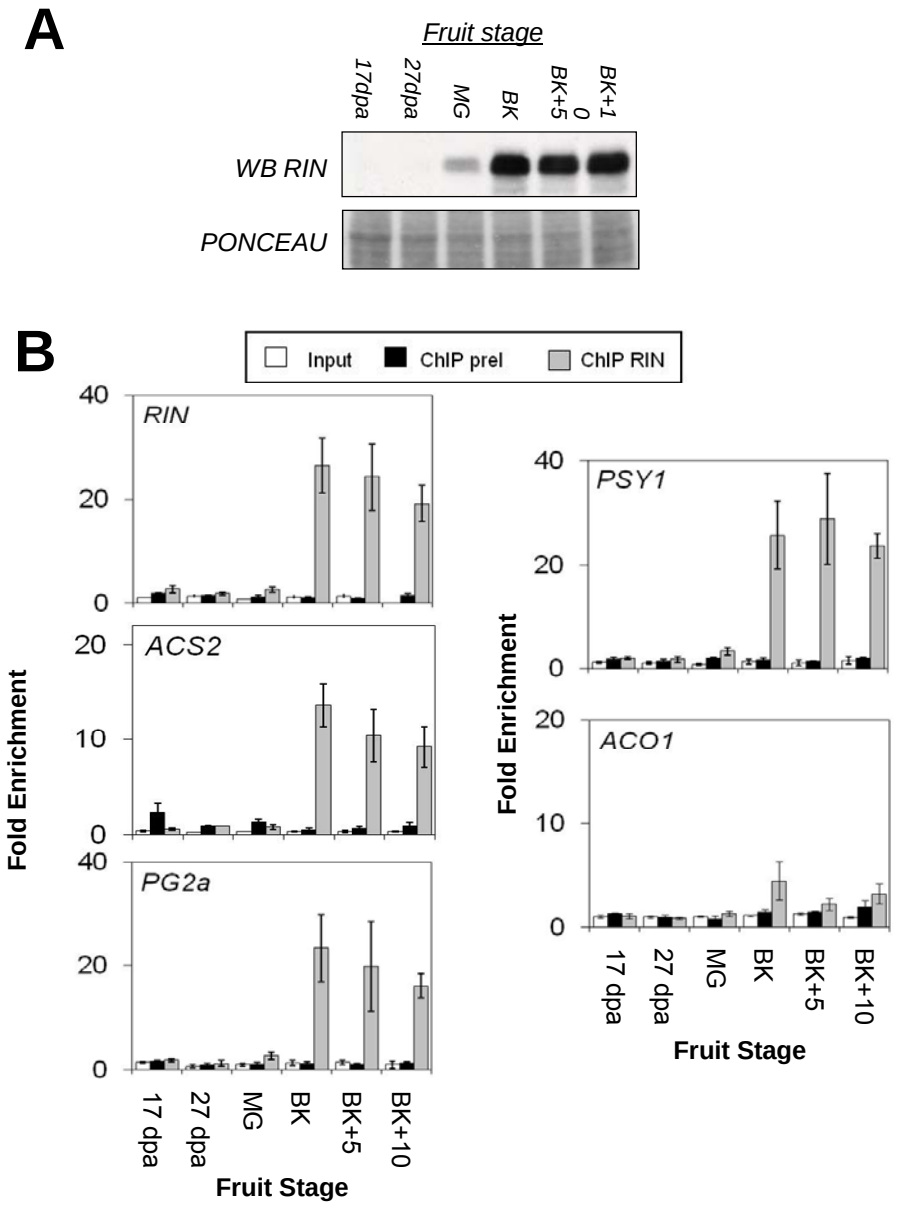


Figure 2-4Time course of RIN-promoter interactions. A) Western blot analysis of cross-linked fruit extract used for chromatin immunoprecipitation using RIN antibodies. B) Chromatin Immunoprecipitation of promoters at different stage of fruit development. Enrichment values correspond to the ratio of gene specific amplification to g18S amplification measured by qPCR.

2.3.4 DNA binding dynamics of RIN during fruit development and ripening

Two models can be proposed regarding the temporal action of RIN in controlling gene expression during fruit ripening. In one, RIN acts during a limited period of time to initiate the expression of a number of target genes, but its presence is not needed once ripening has been initiated. Alternatively, RIN might be required in a continuous fashion to both initiate and maintain the expression of ripening genes. In order to test these hypotheses, I performed ChIP at different stages of fruit development and ripening. One confirmed target gene from each of the four categories tested previously was examined, as well as a non-bound target (*ACO1* promoter). A Western blot analysis (Figure 2-4A) shows the amount of RIN protein present in each of the stages examined. A small amount of RIN protein is detected at the MG stage and strongly detected in the BK stage onward. Figure 2-4B shows that RIN interaction with its target gene is first detected around the BK stage and is maintained for up to 10 days post breaker stage. This result supports the second hypothesis in which RIN activity is required throughout ripening, which is also consistent with the observed RNA and protein expression dynamic of RIN (Figure 2-2).

2.3.5 CNR is required for RIN binding activity

The recent characterization of the mutation underlying the *cnr* phenotype revealed that an epigenic mutation in *SBP-CNR* promoter is responsible for the non-ripening phenotype of this mutant. In order to see if the change in methylation level of the promoter has an effect on RIN binding, the ChIP assay was performed on *cnr* fruits at Bk+2 stage. Interestingly, no enrichment of the *CNR* promoter was observed in *cnr* fruit after the ChIP (Figure 2-5B). I next verified the binding of RIN to its other target genes in the mutant fruit, and found that RIN association with all target genes analysed is lost in the *cnr* mutant (Figure 2-5B).

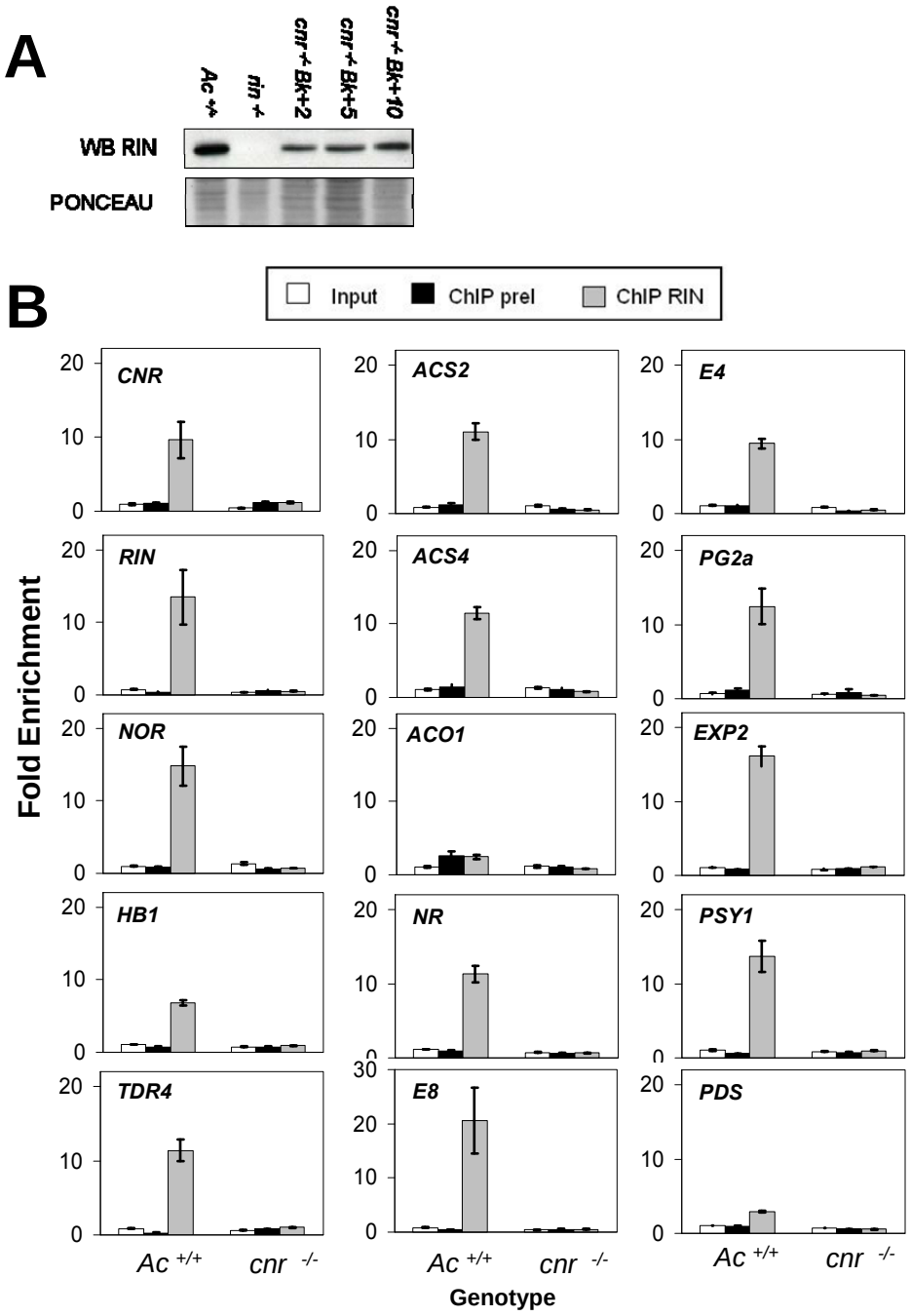


Figure 2-5 CNR requirement for RIN promoter binding activity. A) Western blot analysis using the RIN antibody of proteins extracted from Ac, *rin* and *cnr* fruits. B) Enrichment of *CNR* promoter following RIN ChIP in Ac and *cnr* fruit at the BK+2 stage. C) Enrichment of ripening related promoters following RIN ChIP in Ac and *cnr* fruit at the BK+2 stage

This observation is not due to a lack of RIN protein accumulation in the mutant, as a Western blot analysis of *cnr* mutant tissue clearly demonstrate the presence of RIN protein (Figure 2-5A). Consequently, this result suggests that the RIN protein depends on CNR or a CNR-regulated gene product to efficiently associate with DNA.

2.3.6 Characterization of a RIN binding element

In order to understand how RIN regulates the numerous genes identified, the MEME program (Bailey and Elkan, 1994) was used to search for DNA motifs that are common in RIN-associeated promoters. The most common element found is illustrated in Figure 2-6A. The consensus sequence does not correspond to the typical MADS-box binding site (CArG box) and a subsequent search for similar motifs using the TOMTOM tool (Gupta et al., 2007) did not reveal any similarity with a known binding sites. Fig 2-6B illustrates the position of this motif along with the typical MADS CArG box element in the promoters tested. Interestingly, the MEME motif is found in all, but one of the promoters showing association by RIN and is also absent in the two promoters that do not show any enrichment. On the other hand, no correlation is observed between the presence of CArG boxes and RIN association. This result could indicate that RIN interact with its target gene promoter by association with other transcription factors.

Ito et al. (2008) reported recently the characterization of the RIN binding site *in vitro*. I was not able to repeat their gel retardation experiment using either a full-length or a truncated version of RIN (data not shown). Aside from technical problems, this result could reflect the need for other co-factors to allow efficient DNA binding by the RIN protein.

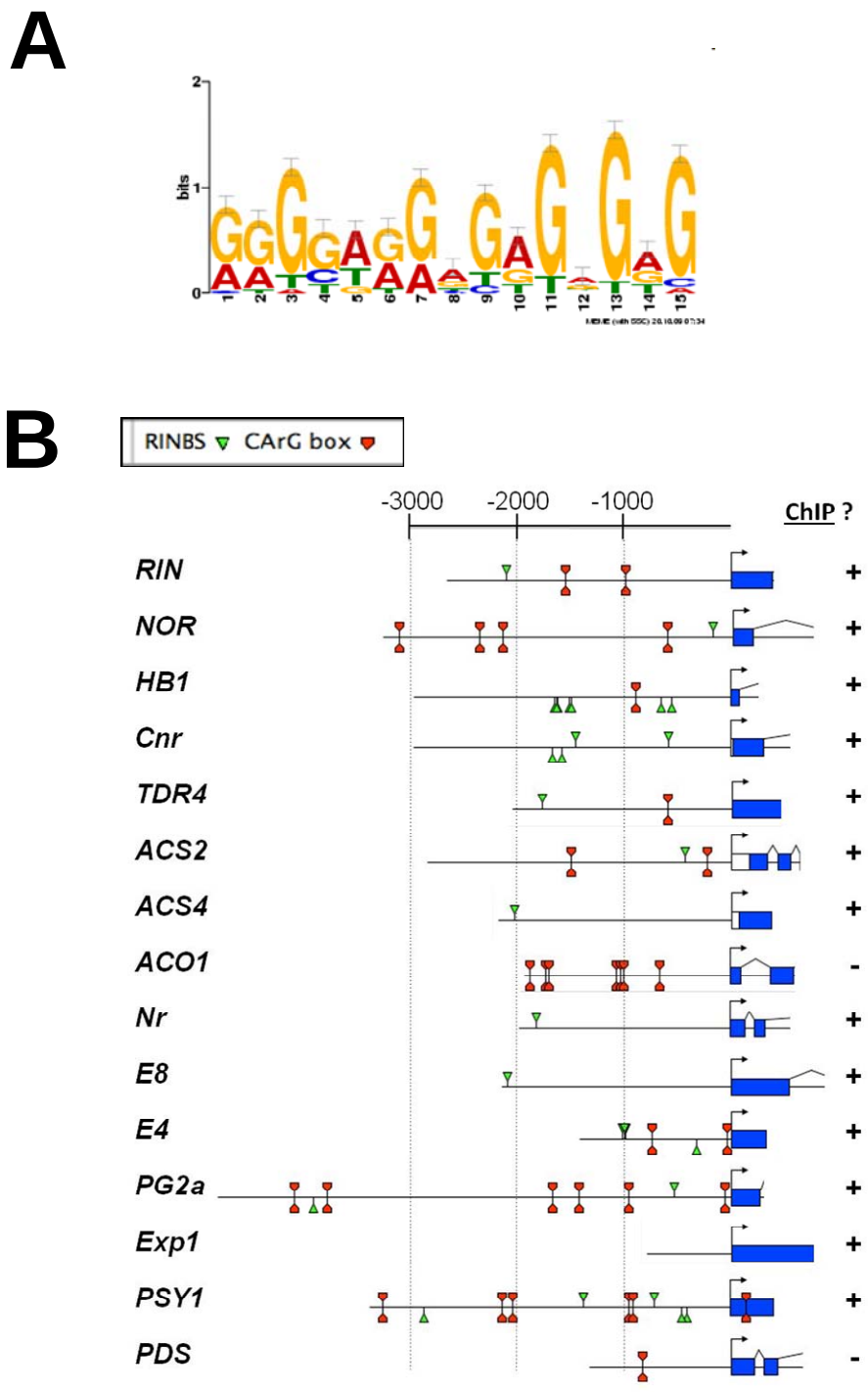


Figure 2-6 Putative RIN motif. A) Result of MEME analysis of promoter immunoprecipitated by RIN. B) Location of MEME-identified motif (motif1) and CArG boxes in the promotors of ripening genes tested in the ChIP assay. The scale is proportional to the number of base pairs upstream of the transcription start site.

2.4 DISCUSSION

The complex phenotype associated with the *rin* mutation suggests that RIN is involved in the regulation of numerous molecular pathways, acting as a master regulator of the ripening cascade. A microarray experiment was performed to identify genes that are deregulated in the *rin* mutant at the onset of ripening. I found 347 genes showing significant deregulation at the BK staged fruit when comparing *rin* to *Ac* fruits. Their identity correlates with the phenotypic observations characterizing the *rin* mutant but the detailed mechanism by which they are regulated by RIN is unclear. Using a chromatin immunoprecipitation approach, I investigated the association of RIN with a subset of ripening-related genes. Our results indicate that RIN can associate with the promoters of numerous genes representing the breadth of ripening phenomena and including transcription factors genes, ethylene synthesis and signaling genes, cell wall modifying enzyme genes, and the rate limiting gene in ripe fruit carotenoid biosyhthesis.

2.4.1 RIN regulation of other transcription factors

The observed binding of RIN to the *NOR* promoter was unexpected since NOR is believed to act upstream of RIN in the ripening cascade (Vrebalov and Giovannoni, unpublished). Indeed, whereas *NOR* transcript is still detected in the *rin* mutant, RIN expression is strongly downregulated in *nor* mutant fruit (Vrebalov, pers. comm.). The binding of RIN to the *NOR* promoter might be involved in strengthening normal *NOR* expression, but might not be necessary for its initial expression. Regulation of a NAC domain transcription factor by a MADS box protein was previously reported by Sablowski and Meyerowitz (1998) who showed that the floral identity dimer AP3/PI directly regulates the NAC domain protein, NAP (Sablowski and Meyerowitz, 1998)

Our result also suggests that a positive feedback loop is involved in *RIN* regulation, since an enrichment of the *RIN* promoter was observed. Several positive and negative autoregulatory feedback loops have previously been reported for several MADS box proteins. Arabidopsis AGL15 was shown to have a direct inhibitory effect on its own expression in embryonic tissue (Heck et al., 1995). Tilly et al. (1998) showed that the expression of *AP3*, a B-class floral identity MADS box gene, is directly influenced by the binding of the AP3-PI dimer to one or more CArG boxes present in its promoter. *PI* gene expression is similarly controlled by the AP3-PI dimer but does not possess a CArG box motif in its promoter (Chen et al., 2000). This latter observation could indicate that the AP3-PI dimer is recruited to the promoter of PI through another DNA binding protein, or could result from indirect regulation. Several other examples of MADS associated regulatory feedback loops have been described (Schwarz-Sommer et al., 1992; Trobner et al., 1992; Hill et al., 1998) and our results indicate that RIN may also operate in such a regulatory loop.

Another interesting observation is the binding of RIN to the *Cnr* promoter. The SBP family is known to be involved in the regulation of the SQUAMOSA MADS box protein; however, little is known regarding their own regulation at the transcriptional level. Our results suggest that RIN, a MADS box protein, could be involved in *CNR* regulation.

2.4.2 RIN and ethylene regulation

A hallmark of climacteric fruit ripening is the production of elevated ethylene. *rin* fruits are unable to produce climacteric ethylene, hence our interest in testing the binding of RIN to ethylene producing genes. Interestingly, both the *ACS2* and *ACS4* gene promoters show enrichment following ChIP, indicating that RIN associates with

their respective promoters. *ACS2* has previously been reported to be regulated by RIN by Ito et al (Ito et al., 2008) and our results further support their conclusion and extend it to *ACS4*. The *ACO1* promoter was not significantly bound by RIN. However *ACO1* has been reported to be regulated by the HD-Zip transcription factor HB1 (Lin et al., 2008) and our result shows that RIN does bind to the promoter of *HB1* therefore connecting RIN and *ACO1* expression.

Promoters of other components of the ethylene signaling cascade associate with RIN, including the ethylene receptor *NR*. This result contradicts data published by Ito et al. (2008) who did not report an enrichment of the *NR* promoter following their RIN ChIP assay. Closer analysis of their data however reveals that the region they tested for enrichment is located in the first intron rather than the upstream sequences of the *NR* gene (AY600437) employed in our analysis.

Another finding of our study is the association of RIN with both the *E4* and *E8* promoters. Confirmation of this association is interesting in light of the numerous studies focusing on the control of their expression during fruit ripening. Both genes were initially cloned based on their rapid response to ethylene in unripe fruits (Lincoln et al., 1987). It has also been reported that although both genes are not induced appreciably by ethylene in the *rin* mutant, exposure of the mutant fruit to exogenous ethylene rescues their expression (Lincoln and Fischer, 1988), suggesting that the reason they are not expressed in *rin* is a direct result of the lack of ethylene production. Numerous studies have focused on the identification of the *cis*-elements responsible for ethylene dependent expression of these two genes (Deikman and Fischer, 1988; Lincoln and Fischer, 1988; Cordes et al., 1989; Dellapenna et al., 1989; Deikman et al., 1992; Xu et al., 1996; Deikman et al., 1998). The picture emerging from these studies is that numerous regions are required for proper expression of both genes. It is also interesting that the regions responsible for ethylene responsiveness in

each promoter seem insufficient to promote the correct high level of expression of these genes during ripening. For example, Deikman et al. (1992,1998) showed that although the region from -1528bp to -1100bp upstream of the transcription start site (TSS) of the *E8* gene is responsible for ethylene responsiveness, this region alone is unable to promote high *E8* expression during normal ripening. Similarly, two regions of the *E4* promoter are apparently required to promote ethylene responsiveness, but fail to do so individually (Xu et al., 1996). A number of studies have demonstrated the binding of the DNA binding protein E4/E8BP to both promoters; however, further analyses have shown that the region bond by E4/E8BP is dispensable for correct expression of the genes during ripening (Deikman and Fischer, 1988; Cordes et al., 1989; Deikman et al., 1998). In light of our results, I believe that RIN is another major and direct mediator of this regulation.

2.4.3 RIN binding motif

MADS box proteins are known to bind to the CArG box motif ($C(A/T)_6 G$) and Ito et al. (2008) characterized a RIN binding site with similarity to this consensus sequence using gel shift assays (Ito et al., 2008). I was unable to repeat their gel shift result and can therefore not confirm *in vitro* binding of RIN to the identified promoter targets. *In silico* analysis using the MEME software was used to identify a common motif found in RIN-bound promoters, which did not correspond to any know *cis*-element. Whether this motif is a *bona fide* binding element remains to be confirmed. The lack of correlation between RIN binding and the presence of CArG motif in a promoter suggest that RIN activity could be mediated by interaction with other DNA binding proteins in, at least some, instances. RIN is a member of the SEP3 clade of MADS box proteins which are known to be involved in the bridging of other MADS box

proteins to form higher order complexes involved in regulating flower development (ABCE model). Similarly RIN could be involved in formation of transcriptional complex through protein-protein interaction with other transcriptional regulators.

2.4.4 Significance of RIN binding

Our ChIP results suggest that RIN binds to a wide variety and large number of ripening-associated genes. Recent work by others have provided data concerning the number of binding sites of transcription factor on a genomic scale using ChIP-sequencing technology, and have shown that transcription factors generally bind to a very large number of target genes. For example, the *A. thaliana* SEP3 protein (a close homolog of the RIN protein) was shown to bind to about 4,000 sites throughout the genome (Kaufmann et al., 2009). Interestingly, only a fraction of the bound regions contained a typical CArG box consensus motif, while many other motifs associated with other known transcription factors were also enriched. Similarly, (Zheng et al., 2009) showed that the MADS box protein AGL15 was bound to 2,000 sites, only 64% of which had a clearly defined CArG *cis* element. The HY5 protein involved in regulating light signal transduction was also shown to bind to more than 3,000 sites (Lee et al., 2007). Considering these studies, the ability of RIN to bind to numerous ripening-associated genes is not unexpected, though the meaning of such global binding remains unclear. An interesting question raised by such a high number of bound target genes is its relation with gene expression. It is becoming clear that binding of a transcription factor to a specific promoter does not immediately activate transcription (Wyrick and Young, 2002). Instead, it is thought that some transcription factors may bind to their targets yet will not affect transcription until other conditions are met (e.g. interaction with other TFs). In line with this model, the expression of

only a minority of AGL15-bound targets are influence by the presence of AGL15 (Zheng et al., 2009). Similarly, the expression of only 6% of HY5 bound targets are affected in a *hy5* mutant (Lee et al., 2007).

Previous studies and our qRT-PCR analysis clearly demonstrate that RIN is required for the regulation of the genes that I have identified by ChIP as target of RIN. However, it is unclear whether RIN binding alone is sufficient to affect expression. Another cofactor might be required for efficient expression of RIN-bound targets. This hypothesis stems from the fact that overexpression RIN in the *nor* mutant background fails to promote the expression of these target genes. This result suggests that another co-factor under the control of NOR is required, together with RIN, to induce ripening-gene expression. A potential partner for this function is the recently described MADS-box gene *TAGL1* (Itkin et al., 2009; Vrebalov et al., 2009). The expression of this gene is independent from RIN and its suppression by RNAi produced a ripening phenotype similar to that of RIN silenced lines (Vrebalov et al., 2002). Furthermore, Itkin et al. (2009) showed that TAGL1 interacts directly with the ACS2 promoter, a gene also bound by RIN (Ito et al., 2008). Here I provide evidence that RIN interacts with numerous ripening gene promoters. The functional nature of the bound genes is known in most cases and suggests that RIN participates intimately in the regulation of numerous ripening genes. These results also point to new transcription factors that may be candidates for ripening regulatory control. Transgenic lines that will facilitate testing of these new hypotheses are currently being produced.

CHAPTER 3 CHARACTERIZATION OF NOR ACTIVITY DURING TOMATO FRUIT RIPENING

3.1 INTRODUCTION

Fruit ripening is the process by which fleshy fruits become attractive to seed dispersing organisms. Ripening generally involves softening of the fruit tissue, conversion of starch to sugar, accumulation of secondary metabolites affecting appearance, taste and aroma (Seymour, 1993). Previous studies on a variety of fruit species have revealed the critical role played by the hormone ethylene in the ripening regulation of so-called climacteric fruits (Barry, 2007). In those fruits which include tomato, stone fruits and banana, ethylene is known to directly regulate the expression of several genes involved in the modification of the fruit tissue. In contrast, the regulation of the ripening in non-climacteric fruit, such as strawberry, citrus and grape, seems to be largely independent of ethylene (Lelievre, 1997). For these fruit the nature of the signal involved in triggering and coordinating ripening is unknown, altough auxin is known to play a crucial role in strawberry fruit maturation (White, 2002).

Tomato (*S. lycopersicum*) has long been used as a model to study climacteric fruit ripening (Giovannoni, 2004). The existence of numerous ripening-defective tomato mutants have helped shed light on the molecular events involved in ripening. The critical role played by ethylene during climacteric ripening was thus elucidated by the study of natural and transgenic ethylene-impaired tomato mutants (Giovannoni 2007). A subset of tomato mutants, including *rin* and *nor*, show a strong inhibition of the ripening phenotype in an ethylene independent manner (Giovannoni, 2007). The fruits

of these mutants fail to ripen and do not respond to exogenous application of ethylene. They have however been shown to possess a fully functional ethylene signaling mechanisms (Vrebalov et al., 2002; Giovannoni, 2007). These mutations are believed to interfere with steps occurring prior to the action of ethylene, including regulation of its biosynthesis. The process whereby fruit become able to respond to ethylene is referred to as ripening competency acquisition as fruit at immature stages do not respond to ethylene by ripening. Ripening competency is believed to be a developmentally regulated stage occurring prior to, though relatively near, the initiation of climacteric ripening (Giovannoni, 2007).

The genes responsible for the *rin* and *nor* mutations each encode members of previously described transcription factor families. The *RIN* gene has been shown to code for a MADS box transcription factor (Vrebalov et al., 2002) and is necessary for the expression of several effector genes (Ito et al., 2008). Interestingly, a functional RIN-like protein is required for the complete ripening of strawberries (a non-climacteric fruit), suggesting that the ripening competency acquisition mechanism is conserved between both climacteric and non-climacteric fruits (Vrebalov et al., 2002 and J. Vrebalov pers. comm.). The *NOR* gene codes for a transcription factor belonging to the plant-specific NAC family (J. Vrebalov, pers. comm.). NAC proteins have been shown to be involved in numerous developmental processes, including establishment of the shoot apical meristem (Souer et al., 1996; Aida et al., 1997; Takada et al., 2001), specification of organ boundaries in both leaves and flowers (Aida et al., 1997; Berger et al., 2009) and secondary cell wall and vascular tissue formation (Mitsuda et al., 2005; Mitsuda et al., 2007; Zhong et al., 2007; Mitsuda and Ohme-Takagi, 2008; Zhao et al., 2010). The exact role of NOR in promoting

ripening remains elusive. The current study aimed to clarify the role of the NOR protein in the early stages of tomato ripening.

3.2 METHODS

Plants

Wt and *nor* (*nor/nor*) mutant tomato plant (cv Ailsa Craig) were grown under normal greenhouse condition until maturity. Fruits were staged based on the number of days from anthesis to breaker stage as defined by the detection of orange coloring at the base of wt fruits.

Microarray

Microarray analyses were performed using the TOM1 oligonucleotide array (Alba et al., 2004; Fei et al., 2004). Fruit mRNA extraction and labeling, microarray hybridization and analyses were performed has described previously (Alba et al., 2004; Alba et al., 2005).

Constructs

pET-NOR FL was obtained by PCR amplifying the full length *NOR* cDNA using primers NOR F (5'-TTTT**CTCGAG**TTAAGAGTACCAATTCATGCC -3') and NOR R (5'-TTTTGGATCC**GAATTC**ATGGAAAGTACGGATTCATC-3') and pSK-NOR as template (J. Vrebalov, unpublished). The PCR product was digested with *EcoRI* and *XhoI* enzymes and cloned into pET28a vector (Novagen) previously digested with the same enzymes. The pET-NOR-C was obtained by all-around PCR amplification of pET-NOR FL using primers NORC-F (5'-TATAGGTACCTTG-AGGCTAGATGATTGG -3') and pET28a R (5'-TATAGGTACCCATTTG-CTGTCCACCAGTC-3'). The PCR product was digested with *KpnI* and *DpnI* restriction enzymes (NEB) and self ligated.

Recombinant protein purification and Antibody production

The pET-NOR C plasmid was introduced in *E.coli* BL21 Star (DE3) cells (Invitrogen) and recombinant His-NORC protein purified as described previously (see Chapter 2). Briefly, HIS-tagged NORC was purified from inclusion bodies under denaturing conditions using the TALON His Batch/Gravity-Flow Column purification protocol (Clontech). 1mg of purified His-NOR-KC protein was sent to Covance Research Products (Denver, PA) for injection into rabbits to raise antibodies.

Protein extraction from fruit and Western blot analysis

Proteins were extracted from fruit as previously described (see Chapter 2). 25µg of each protein extract was separated by SDS-PAGE (10%) for analysis. Following electrotransfer to nitrocellulose membrane, NOR protein was detected using a 1:1,000 dilution of the rabbit anti-NOR antibody and a 1:100,000 dilution of the HRP-coupled rabbit secondary antibody (Sigma).

Chromatin Immunoprecipitation +qPCR

Chromatin immunoprecipitatons were performed according to the protocol described in Chapter 2. Quantitative PCR of immunoprecipitated DNA was performed using SYBR Green on the AB 7900 platform following manufacturer's instructions. Briefly, 2µl of gDNA sample obtained as described above was mixed with 5µl of SYBR Green and 300nM of promoter-specific primers. Relative fold enrichment was calculated by dividing the amount of gene specific amplification by 18S amplification. Table 3-1 lists the primers used in the qPCR reactions.

qRTPCR

RNA was extracted from ground frozen fruit tissue at different stages of fruit development using Plant RNA purification solution (Invitrogen) and following manufacturer's instructions. RNA was then digested with RQ1 DNase (Promega), and further purified on Qiagen RNA purification columns. Quantitative RT-PCR was performed using SYBR Green on the AB7900 platform using 18S rRNA as the

internal control. Gene specific primers used in the qRT-PCR assay are listed in Table 3-1.

Electromobility shift assay

Protein extracts containing recombinant His-NOR protein was obtained by expression of the pET-NORFL construct in *E. coli* Bl21, solubilization of inclusion bodies and renaturation (Perry et al., 1996). Probes were generated by first annealing complementary primer pairs containing the sequences of interest and labeling the double stranded DNA with ^{32}P-ATP using T4 polynucleotide kinase (NEB). Cell extracts containing the renatured inclusion bodies were incubated with the labeled probes as described in (Wang et al., 2002).

Bioinformatics

Promoter sequences of candidate genes were obtained from the SGN website (http://solgenomics.net). Searches for cis-elements within promoter regions were performed using the Regulatory Sequence Analysis Tools (RSAT) (http://rsat.ccb.sickkids.ca).

Table 3-1 Primers used for gene expression (qRT-PCR) and Chromatin Immunoprecipitation (qPCR) enrichment measurements

Gene	Acc. no	Primer sequences (5'-3')	
		GENE EXPRESSION	CHIP
PG2a	X04583 X80908	F: TCAAGGGCACAAGTGCAACAAAGG R: TGCACGTAGCCTCTGATGGTTT	F GGATGCAGGGTTGTACAAAGAGAG R AGTCGTCGTATGAAGGCGAAAGGA
ACO1	X58273	F: TGGAGATGAGAGAGCCAACA R: TTCCATGGTTCACCAACTCA	F: GGCATGTTCCTTCTAACTTAATTAGC ATTC R: TGAGAGGTTCACAAATTCTCCCTC
NR	AY600437	F: ATCAGGTTGCTGTCGCTCTT R: GGCCATCTCTGCTTCTTGTC	F: GGGTACTATGTCATGTTCCGTCAC R: TCTAGTGTCTTCCACTAACCTCCC
E8	DQ317599 X13437	F : T G G C T C C G A A T C C T C C C A G T C T R: GTCCGCCTCTGCCACTGAGC	F: CTGCACAAGACTTTCTATTGTTG GG R: ACGTCTAGAAAGACCCATATCT
RIN	AF448522	F : CATGGCATTGTGGTGAGCAAAG TGT R : AGCATCATGTGTTGATGGTGCTGC	F : CCATCCTGTTAGTGATATTGTCT GC R : TGAAGTGTACTGACATTCCGGT

3.3 RESULTS

3.3.1 Characterization of the NOR protein

In order to study the role of NOR *in planta*, I developed an antibody that would specifically recognize this protein. Since the N-terminal NAC domains of most members of the NAC family are highly similar, I raised the antibody against the more variable C-terminal portion of the NOR protein. A His-tagged version of the C-terminal region of the NOR protein was expressed in *E. coli*, purified on a metal-affinity column and injected into rabbits. The specificity of the polyclonal antibody obtained from the rabbit serum was tested by Western blot analysis of total protein extracts isolated from wt and *nor* mutant BK stage fruits. Based on the amino acid sequence (355 aa), the estimated mass of NOR is 39 kDa . The *nor* mutation

introduces a stop codon in the middle of the NOR open reading frame that should lead to the production of a truncated protein lacking the C-terminal domain, whether the truncated protein is stable or not remains unclear. In either case, the NOR antibody should not detect any protein, since it is directed toward the C-terminal portion of the NOR protein, a region that is not expected to be translated from the mutant allele. Figure 3-1A shows that the NOR antibody specifically recognized a 40 kDa protein in the *wt* fruit but not in *nor* fruit. This protein is further detected in a complementation line expressing a *NOR* transgene under the control of the 35S promoter in the *nor* mutant background. This result strongly suggest that the NOR antibody is highly specific.

3.3.2 NOR protein accumulation during fruit development

I used the NOR antibody to examine the accumulation behavior of the NOR protein during fruit development and ripening. As expected from the RNA expression patterns, NOR protein starts to accumulate in the pericarp of tomato fruit early after the MG stage (Figure3-1B). Proteins and RNA reach their highest levels around the BK stage and then gradually decline during later ripening. This expression pattern is consistent with the proposed role of *NOR* in initiating early steps of the ripening cascade.

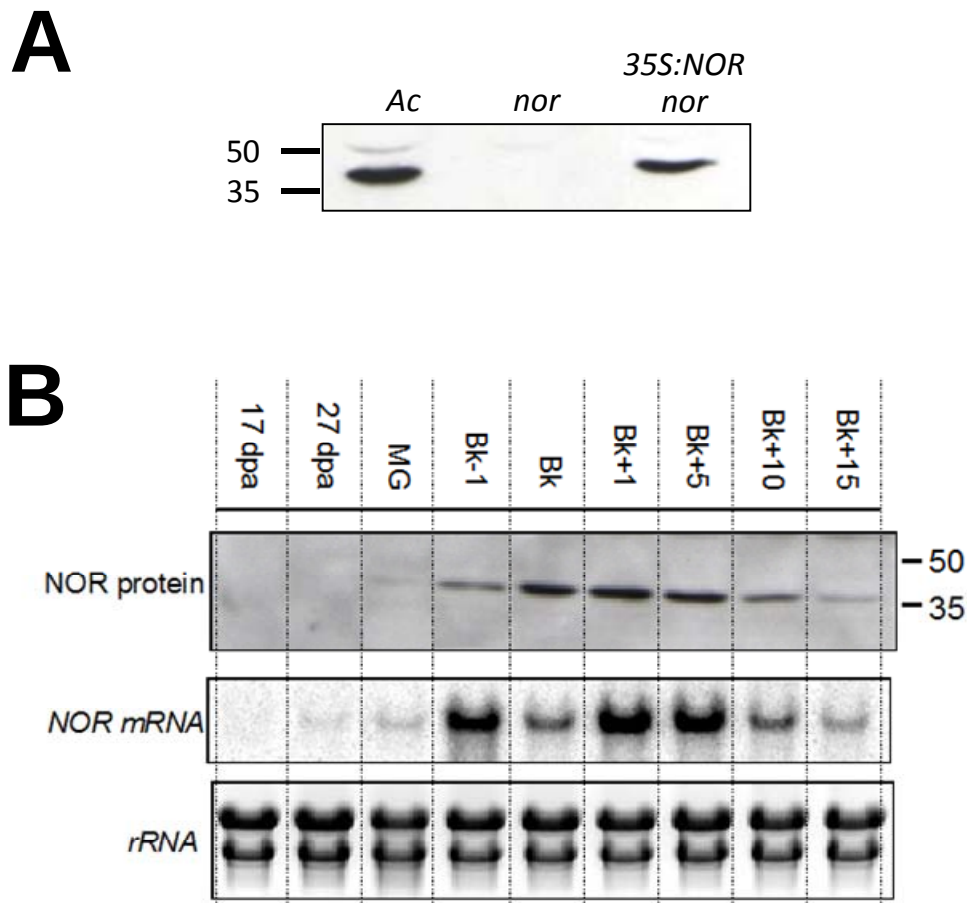


Figure 3-1 NOR specific antibody A)Western blot analysis of fruit protein extract from Ac wt, *nor* and complementation line 35S:NOR in *nor*. Western blotting was performed using the NOR antibody. B) Time-course of NOR protein and mRNA accumulation during fruit development and ripening. Fruit protein and mRNA were extracted from wt Ac fruits collected at different time points as indicated and detected using Western blot analysis (NOR antibody) and Northern blot analysis. dpa = days post anthesis.

3.3.3 NOR specifically binds DNA in vitro

NAC domain proteins were previously shown to bind to a specific DNA binding motif known as the NAC Binding Site (NACBS) (Olsen et al., 2005). In order to test if the NOR protein can also bind to this motif I performed a gel-shift assay using recombinant His-NOR protein. Most of the recombinant His-NOR protein is localized

in insoluble inclusion bodies. Inclusion bodies were first solubilized under denaturing conditions and then renatured to obtain functional NOR protein (see Material and Methods). The renatured NOR protein extract was then incubated with a radioactively labeled double-stranded oligonucleotide containing a single (1XNACBS) or duplicated (2XNACBS) copy of the NACBS (Olsen et al., 2005) (Figure 3-2A). Figure 3-2B shows that the NOR containing extract efficiently binds to the 2XNACBS probe whereas an *E. coli* extract lacking the NOR protein fails to do so. The NOR containing extract no longer binds to a probe containing point mutations which destroy the consensus motifs ($2XNACBS^{mut}$). This indicates that NOR can interact *in vitro* with a dual NACBS motif. Interestingly, no interaction could be detected between the NOR extract and the 1XNACBS probe even when the amount of NOR extract was increased 10 fold relative to the amount sufficient to bind the 2XNACBS probe. This indicates that NOR has a much lower affinity, if any, for single NACBS *in vitro*.

3.3.4 Identification of genes regulated by NOR

The severe non-ripening phenotype associated with the *nor* mutation suggests that many biochemical pathways are influenced by this gene. In order to get a more detailed picture of the genes affected by the mutation, a microarray experiment was performed using the cDNA array Tom1 (Alba et al., 2004; Fei et al., 2004). I compared cDNA populations derived from *wt* and *nor* BK fruit to those of MG *wt* fruit (Figure 3-3A). Four biological replicates, including 2 dye swaps, were performed for each comparison. A total of 719 microarray features corresponding to 450 unigenes showed significantly altered expression levels (>2fold, $p<0.05$) in *nor* BK fruit compared to *wt* BK fruit. Among those, 258 genes were up-regulated and 192 were

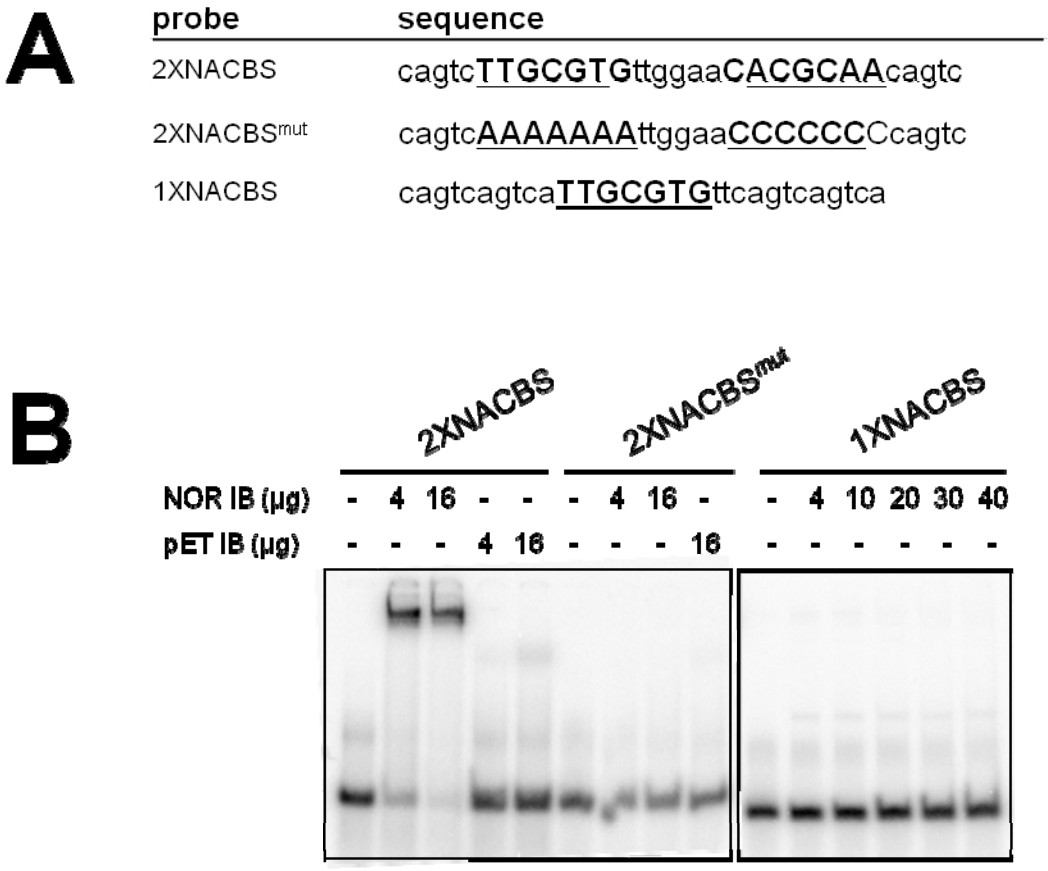


Figure 3-2 NOR DNA binding activity A) Sequence of the oligonucleotides probes in gel shift assays. NACBS motifs are shown in bold capital letters. B) EMSA. *E.coli* inclusion bodies containing the recombinant NOR protein (NOR-IB) or a control vector (pET IB) were incubated with double stranded radioactive probe as noted above the respective lanes. The amount of protein extract used for the assay is expressed in µg.

down-regulated. Fig 3-3B depicts the expression levels of those 719 features in the original comparison of the BK and MG stage fruit. Table 3-2 lists the distribution based on Gene Ontology (GO) annotation of the 450 unigenes. This approach identified several genes known to be involved in ripening, including the *NOR* gene itself, whose expression is strongly reduced in the *nor* mutant (Table S3-1).

In order to confirm the microarray data, qRT-PCR of *nor* and *wt* fruits at MG and BK stages was performed on a subset of identified genes. Fig 3-3D shows that the

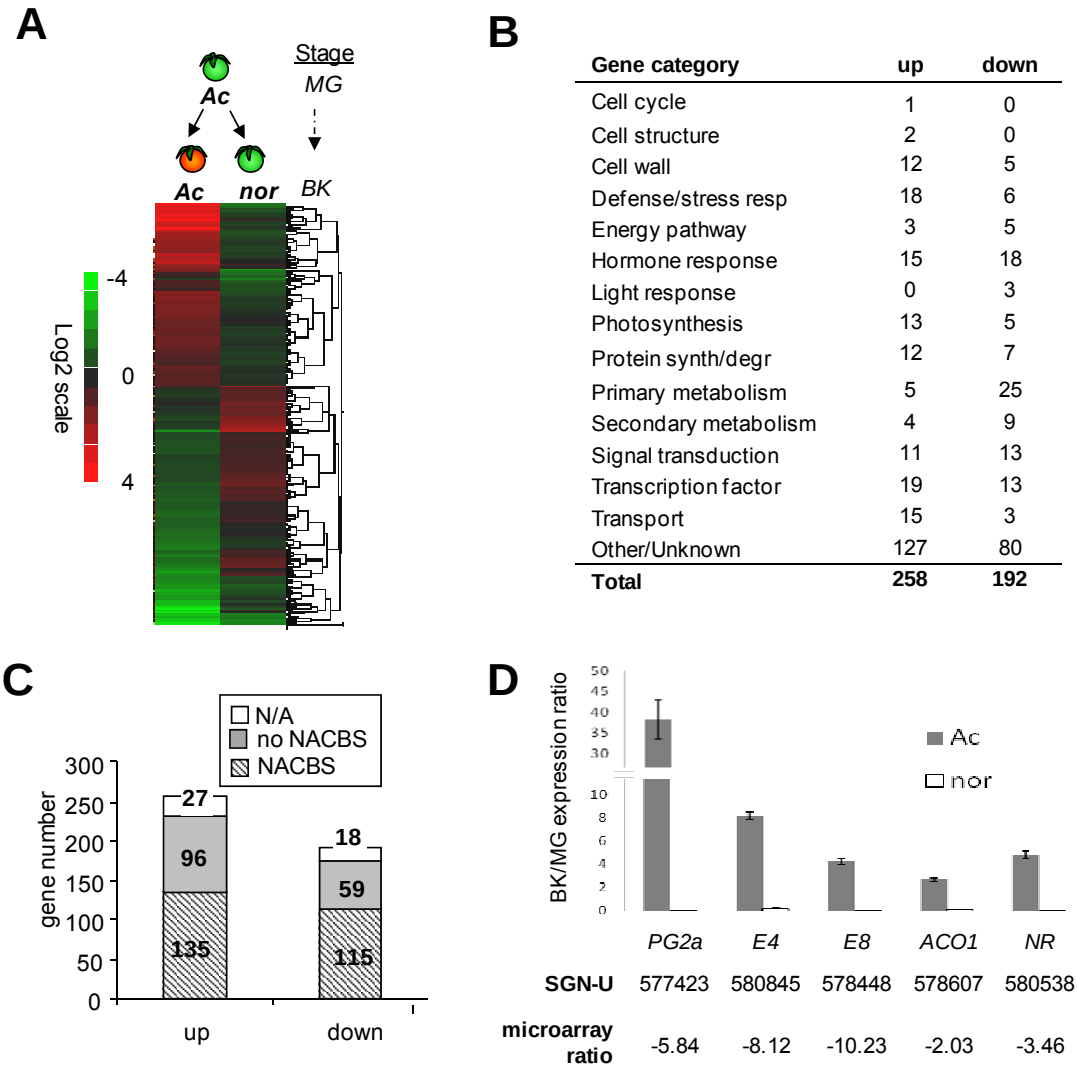
expression profiles of *PG2a, E8 , NR* and *ACO*1 are strongly reduced in the *nor* mutant at the BK stage as compared to wt. The results correlate with the microarray data thus confirming the general validity of the latter.

3.3.5 Presence of NACBS in microarray-identified genes

I searched for the presence of the NACBS in the promoter sequence of unigenes identified in our microarray and found that none of the 432 unigenes for which a promoter sequence is available possesses a duplicated NACBS (defined as the sequence TTnCGTRN(2,20)RACGNAA). However, 250 unigene promoters contain at least one single NACBS (TTnCGTR). The distribution of NACBS among up and down regulated genes is shown in Figure 3-3 C.

3.3.6 *In vitro* binding of NOR to NACBS sites found in target genes

I next tested whether NOR could interact directly with the NACBS of some of the microarray-identified target genes. Figure 3-4A shows the location of NACBS *cis* elements in the promoter of the *ACO1*, *NR*, *E8*, *PG2a* and *RIN* genes. To assess NOR-binding *in vitro,* I conducted a gel retardation assay using double-stranded oligonucleotide probes corresponding to the three NACBS motifs of *E8*, the NACBS motif of *Nr*, and the NACBS motif of *RIN* promoters. As shown in figure 3-4B, a clear shift is observed for *E8* NACBS3, but not for the other two *E8* NACBS motifs. A faint but reproducible band is also detected for *NR* NACBS, but not for the RIN motif. This result shows that NOR bind to single NACBS site present in NOR-dependent ripening genes *in vitro*.



Gene category	up	down
Cell cycle	1	0
Cell structure	2	0
Cell wall	12	5
Defense/stress resp	18	6
Energy pathway	3	5
Hormone response	15	18
Light response	0	3
Photosynthesis	13	5
Protein synth/degr	12	7
Primary metabolism	5	25
Secondary metabolism	4	9
Signal transduction	11	13
Transcription factor	19	13
Transport	15	3
Other/Unknown	127	80
Total	**258**	**192**

Figure 3-3 nor fruit transcriptome profiling . A) Heat map showing expression level of TOM1 microarray features in Ac and *nor* fruits compared to Ac MG fruits. Only genes showing significant ($p<0.05$) differential expression (>2fold) between BK stage Ac and *nor* fruit are depicted. B) Distribution of up and down regulated genes between *nor* and Ac BK fruits based on their gene ontology (GO). C) Distribution of putative NACBS sequences in the promoter of up and down regulated unigenes identified in the *nor*/Ac BK microarray comparison. D) qRT-PCR of selected unigenes. Expression level of Ac and *nor* BK fruit normalized to the expression level at the MG stage of Ac. Microarray enrichment levels are listed below the SGN identifiers.

3.3.7 NOR associate with the promoters of putative target genes

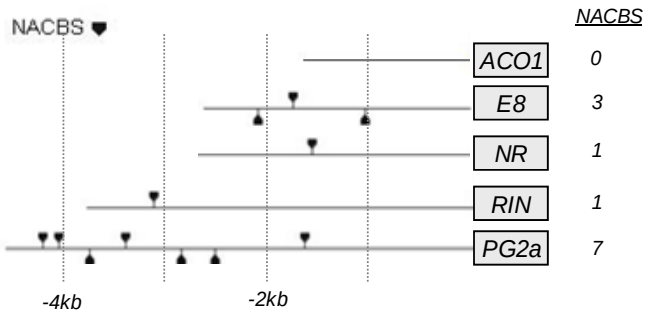
In order to test if the *in vitro* binding of NOR to certain NACBS sequences in the promoters of candidate genes occurs *in vivo*, a chromatin immunoprecipitation strategy was undertaken. For that purpose, BK fruits from *nor* and *wt* plants were cross-linked, their chromatin sheared and subjected to an immunoprecipitation using either a rabbit preimmune serum, or the serum containing the NOR antibody described above. The immunoprecipitated chromatin was then analyzed by qPCR in order to detect levels of enrichment for each promoter following the ChIP assay. Figure 3-4C shows that the *PG2a*, *E8* and *NR* promoters are enriched following the NOR immunoprecipitation only in wt BK fruits. Neither *ACO1*, whose promoter does not contain a NACBS site, or *RIN* whose NACBS sequence was unable to bind to NOR *in vitro*, show enrichment. The ChIP results strongly suggests that NOR can influence the transcription of *PG2a*, *E8* and *NR* genes *in vivo* by interacting with their respective promoters.

3.4 DISCUSSION

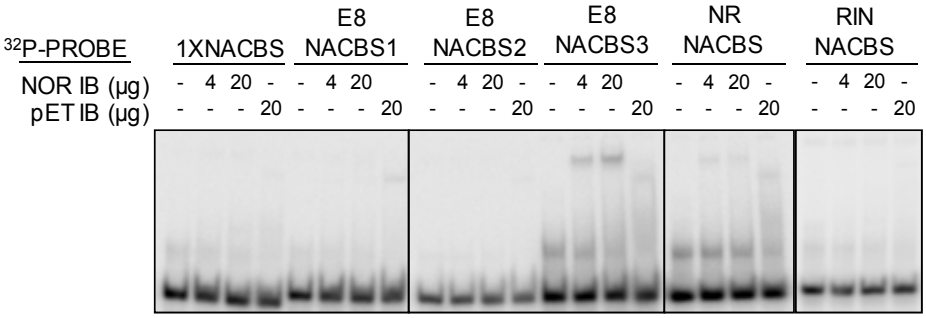
The *NOR* gene plays a critical role in tomato fruit ripening, as illustrated by the dramatic non-ripening phenotype observed in the *nor* mutant (Tiegchellar et al., 1974). *NOR* belongs to the NAC family of transcription factors whose members play important regulatory roles in numerous developmental programs (Olsen et al., 2005). The aim of the current study was to characterize in more detail the functional properties of the NOR protein in order to gain insight into its role during fruit ripening. Using protein assays and a microarray approach, I showed that NOR influences the expression of several hundred genes during fruit ripening and is able to associate with the promoters of some, possibly through interaction with NACBS *cis*-elements.

Figure 3-4 NOR binding to ripening promoters. A) Diagram of the promoter region of selected genes with location of putative NACBS (TTnCGTR) sequences. B) EMSA. *E.coli* inclusion body extracts containing the recombinant NOR protein (NOR-IB) or a control vector (pET IB) was incubated with double stranded radioactive probe as noted. The amount of protein extract used for the assay is expressed in µg. Sequences of the probes are given. C) Chromatin immunoprecipitation assay using the NOR antibody. Enrichment corresponds to the ratio of gene specific amplification to g18S amplification, measured by qPCR

A

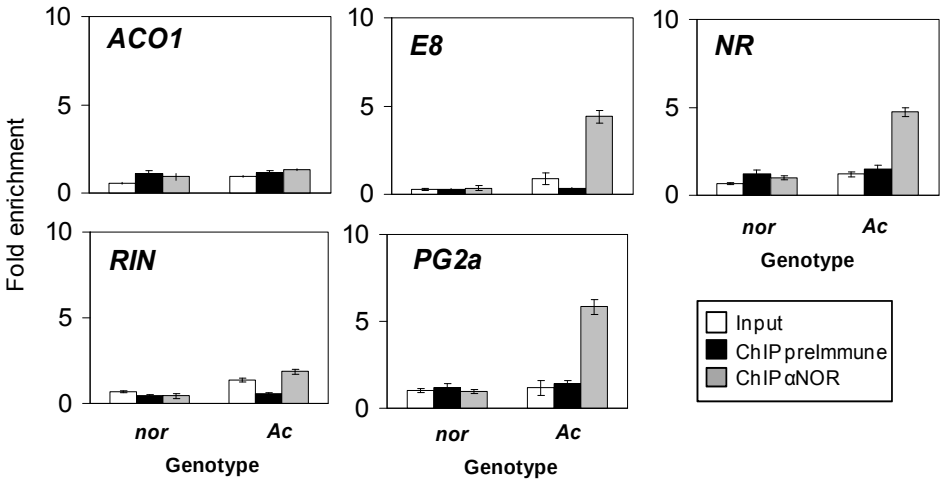


B



probe	sequence
E8 NACBS1	cttcacttaactt**TTGCGTA**aataaaacgaatt
E8 NACBS2	taatgatattgtt**CACGTAA**ttaagttttgtg
E8 NACBS3	tagaaggaatttt**CACGAAA**ttcggcccttattc
NR NACBS	tagatgacac**TTACGTA**tgccgactcaaatt
RIN NACBS1	gcaatacttttt**TTTCGTC**agaagaaatgtt

C



3.4.1 NOR protein accumulates early during ripening

I generated a polyclonal antibody that specifically recognizes the NOR protein in fruit. Using this antibody I have shown that the NOR protein starts to accumulate very early in ripening, being detected before the BK stage (Fig3-1B). This result is in agreement with the hypothesized role of *NOR* in regulating the onset of the ripening program. I also noted that the pattern of NOR protein accumulation during fruit development and ripening correlates tightly with its corresponding RNA profile. This suggests that the protein has a relatively short half-life. Levels of other NAC domain proteins are known to be regulated by post-transcriptional and post-translational mechanisms including miRNAs (Chen, 2004; Mallory et al., 2004; Berger et al., 2009; Larue et al., 2009) and ubiquitin-mediated degradation (Xie et al., 2002; Greve et al., 2003). It would be interesting to determine whether a similar mechanism is involved in the regulation of NOR protein during ripening.

3.4.2 NOR can bind to NACBS consensus sequences in vitro

In vitro gel shift assays confirmed the ability of NOR to bind to the duplicate, palindromically arranged NACBS motif TTgCGTg (Olsen et al., 2005). Interestingly, NOR was not able to bind to a single copy of this motif *in vitro*. Binding of ANAC019 has been observed with this motif (Olsen et al., 2005), but with a much weaker affinity than that observed for the double NACBS motif. Lower affinity for a single NACBS motif is explained by the stochiometry of the NAC complex. NAC proteins bind DNA as dimers and their preferred binding site therefore consists of two closely positioned NACBS sequences. The rare occurrences of such double NACBS sites in actual plant promoters (Olsen et al., 2005) suggests that the binding preference of NAC dimers *in vivo* is less stringent then what is observed *in vitro*, possibly as a result of interaction with other proteins (Tran et al., 2007). In addition to lower

affinity for the single motif, lack of NOR binding to the 1XNACBS used in the current experiment may be due to the specific NACBS sequence employed. I used the ANAC019 preferred motif to test NOR binding to both single and double NACBS. It is possible that the preferred sequence bound by NOR is slightly different than that used in our experimental system. Previous reports have shown that each of the NAC proteins show slightly different affinities for the sequences flanking the core CGT of the motif (Tran et al., 2004; Olsen et al., 2005; Kim et al., 2007; Tran et al., 2007; Ogo et al., 2008). The fact that NOR was able to bind *in vitro* to one of the NACBS of the *E8* promoter and to the *NR* NACBS demonstrates the ability of NOR to bind single NACBS promoters and gives some information regarding NOR motif preferences. Further experiments, such as cyclic amplification and selection of targets (CASTing) (Pierrou et al., 1995), would be useful to determine the exact motif preferred by NOR and to identify additional putative target genes.

3.4.3 Microarrays confirm the expression of many genes is influenced by NOR during ripening

By comparing the mRNA profile of BK-staged *nor* and *wt* fruit, I showed that the *nor* mutation influences the expression of at least 450 genes. A closer examination of the identity of these genes reveals many interesting details about the mechanisms and pathways influenced by *NOR*.

Climacteric ripening fruits adjust their metabolism at the onset of the ripening program to allow for many changes to occur including major increases in non-photosynthetic energy production (glycolysis, fatty acid ß-oxidation) and changes in the level of metabolites that will form the basic building blocks of the numerous compounds present in ripening fruits (e.g. ethylene, carotenoids, flavonoids, ascorbate, cell wall polysacharides, aroma volatiles). The large number of genes involved in

amino acid, carbohydrate, lipid and nucleotide metabolism that are down regulated in the *nor* mutant reflects the failure of *nor* fruits to undergo the normal metabolic transition necessary to support ripening. Among the downregulated genes are several lipoxygenases (SGN-U572041, SGN-U577893,SGN-U578028) , including the previously characterized *TomLoxC* (Heitz et al., 1997; Chen et al., 2004). Lipoxygenases catalyze the dioxygenation of polysaturated fatty-acids and are known to play an important role in the generation of numerous aromatic compounds found in ripe fruits (Heitz et al., 1997; Chen et al., 2004; Leone et al., 2006; Kalua and Boss, 2009; Kovacs et al., 2009; Zhang et al., 2009). Changes in carbohydrate metabolism are responsible for the efficient conversion of starch to soluble sugars during ripening. Our microarray analyses identified the enzyme tomato acid invertase1 (TIV1) (SGN U578195) among those genes downregulated in the *nor* mutant. TIV1 has been shown to influence the ratio of hexose to sucrose in ripe fruits (Klann et al., 1992; Klann et al., 1996). Several amino acid metabolic enzymes were also indentified, including the peptide methionine sulfoxide reductase-like gene *E4* (SGN-U582407), which is believed to be involved in the ethylene biosynthesis; the histidine decarboxylase gene *hdc* (SGN-U580826) (Picton et al., 1993); and gene encoding cysteine, serine and tryptophan modifying enzymes (SGN-U582407, SGN-U576881, SGN-U58541), whose role during fruit ripening remains unclear.

Another interesting observation from our transcriptome analysis is the high number of genes encoding transcription factors that are both up and down regulated by the *nor* mutation. This suggests that NOR may act as a major regulatory switch for the transcriptional cascade network involved in ripening. Although the numbers of transcription factor genes that are up and down regulated in the *nor* mutant are similar to each other, a closer examination of their identities reveals some interesting features. A large number of up regulated genes are members of the *WRKY* family of

transcription factors, which comprise a large families in plants that are involved mainly in biotic and abiotic stress responses and seed germination (Rushton et al., 2010). A role for a *WRKY* gene during fruit ripening has not yet been demonstrated. Our analysis also revealed that numerous defense response genes are up-regulated in *nor* mutant fruits compared to wt controls and it would therefore be interesting to determine whether the normal NOR-dependant down regulation of WRKY transcription factors at the onset of ripening plays a role in the increase pathogen susceptibility of ripe fruits. Another interesting finding among *nor* mutant up-regulated transcription factors, is the presence of the *NAM-like* gene (SGN-U568609). *NAM-like* is the closest known homolog of *NOR* (Yang et al., 2010) and is up regulated during normal tomato ripening. I suggest that the up regulation observed in the *nor* mutant may be the result of a direct regulation by NOR or the result of a feedback mechanism to compensate for the absence of NOR. Three other *NAC* genes (SGN-U568605, SGN-U567211, SGN-U568610) are downregulated in the *nor* mutant and, since NAC proteins form homo- and heterodimers it may be that these genes are candidates for additional mediators of fruit ripening. Another transcription factor which is positively regulated by NOR is the MADS-box gene *TDR4* (SGN-U577950) a homolog of which from *V. myrtillus* was recently shown to be involved in anthocyanin accumulation in bilberry fruits (Jaakola et al., 2010). The role played by this protein during tomato ripening remains to be determined.

A significant number of hormone-related genes are found to be up and down regulated in the *nor* mutant and their identity sheds some light on processes operating in the early stages of ripening. Indeed, almost all the hormone-related genes down regulated by the *nor* mutation are involved in ethylene synthesis or perception (*ACS2, ACO1, E8, E4, NR*). This is consistent with *NOR*'s role in the regulation of ethylene synthesis and the switch between system1 and system2 of ethylene perception (Lin et al., 2009).

Interestingly, several auxin-related genes are up regulated in the *nor* mutant. Auxin is known to play several roles during different phases of fruit development: high auxin levels are correlated with increased fruit set, parthenocarpy and cell expansion (Gustafson, 1936, 1939; Mapelli and Lombardi, 1982; Gillaspy et al., 1993). Accordingly, the auxin-resistant tomato mutant *diageotropica* (*dgt)* has reduced fruit set and fruit size (Balbi and Lomax, 2003). Interestingly, time from anthesis to breaker is significantly increased in the *dgt* mutant, suggesting a role for auxin is regulating the onset of ripening (Balbi and Lomax, 2003). Our microarray analysis also suggests that NOR may be an important regulator of the cross-talk between ethylene and auxin signaling during ripening. I hypothesize that the increased expression of auxin-related genes observed in the *nor* mutant is caused by the existence of a feedback loop triggered by the failure of the fruit to initiate the ripening program as monitored by ethylene production. Consistent with this idea, the F-box gene *SlEBF1* and *2*, which are negative regulators of ethylene response, are down regulated by auxin and up-regulated by ethylene (Yang et al., 2010). Similarly, the tomato auxin response factor *DR12* is negatively regulated by ethylene during the early ripening stages (Jones et al., 2002).

3.4.4 Direct regulation by NOR

The influence of NOR on the expression of the 450 genes identified by microarray analysis could be mediated by either a direct recruitment of NOR to the promoter of these genes, or indirectly through NOR regulation of other transcription factors. I tested the ability of NOR to directly interact with the promoter of some of these genes both *in vitro* and *in vivo*. Based on our results, the presence of an *in vitro* NOR bound NACBS in the promoter of a gene is strongly correlated with *in vivo* binding. Thus binding of NOR *in vivo* was detected for *E8* and *NR* (each contains one NACBS

bound by NOR in our gel shift assay). In contrast, NOR did not bind to the NACBS motif present in the RIN promoter, a result consistent with the absence of *in vivo* interaction in our ChIP assay. Further experiments will be required to determine the identity of other primary targets of NOR. A better characterization of the preferred binding site of NOR has begun to emerge from these experiments and will assist *in silico* identification of additional candidate genes.

3.4.5 Conclusion

Many NAC proteins have been shown to act as master regulators of transcription factor networks (Nakashima et al., 2007; Balazadeh et al., 2010; Seo et al., 2010; Yamaguchi et al., 2010). The work presented here provides important details regarding the mechanism by which NOR acts a major regulatory hub for tomato fruit ripening. NOR interacts with a number of previously characterized ripening genes and either directly or indirectly influences many others. The large number of transcription factors shown here to be influenced by NOR suggests a substantial component of this regulation may be mediated via additional downstream regulators.

APPENDIX : SUPPLEMENTARY DATA

Table 3-S1 Upregulated genes (>2X, p<0,05) *nor* vs *wt* fruits at the BK

SGN	*ID/best blast*	*Gene Ontology*
SGN-U573964	prephenate dehydratase family [Arabidopsis thaliana]	amino acid metabolism
SGN-U577168	Histidine decarboxylase (HDC) (TOM92)	amino acid metabolism
SGN-U578034	pyruvate dehydrogenase E1 beta subunit isoform 1 [Zea mays]	carbohydrate metabolism
SGN-U579539	alpha,alpha-trehalose-phosphate synthase, UDP-forming , putative [Arabidopsis thaliana]	carbohydrate metabolism;
SGN-U579802	Cell division protein ftsH homolog, chloroplast precursor (DS9)	cell cycle
SGN-U581194	INDUCED STOLEN TIP PROTEIN TUB8	cell structure
SGN-U578499	alpha-tubulin [Gossypium hirsutum]	cell structure
SGN-U579510	alpha-tubulin [Nicotiana tabacum]	cell structure
SGN-U576260	Pectinesterase 3 precursor (Pectin methylesterase 3) (PE 3)	cell wall
SGN-U568740	pectinesterase like protein [Arabidopsis thaliana]	cell wall
SGN-U592942	syringolide-induced protein 19-1-5 [Glycine max]	cell wall
SGN-U581990	syringolide-induced protein B13-1-1 [Glycine max]	cell wall
SGN-U578224	xyloglucan endo-1,4-beta-D-glucanase (EC 3.2.1.-) precursor (clone tXET-B1) - tomato	cell wall
SGN-U577260	xyloglucan endotransglycosylase LeXET2 [Lycopersicon esculentum]	cell wall
SGN-U579445	syringolide-induced protein 19-1-5 [Glycine max]	cell wall
SGN-U567218	cellulose synthase-like protein D4 [Populus tremuloides]	cell wall
SGN-U571690	cellulase (EC 3.2.1.4) Cel3, membrane-anchored - tomato	cell wall
SGN-U577484	arabinogalactan protein [Nicotiana alata]	cell wall
SGN-U584639	extensin homolog - potato	cell wall
SGN-U583056	expansin-like protein [Quercus robur]	cell wall
SGN-U563624	tospovirus resistance protein B [Lycopersicon esculentum]	defense response
SGN-U577666	Avr9/Cf-9 rapidly elicited protein 75 [Nicotiana tabacum]	defense response
SGN-U580954	Elicitor inducible gene product Nt-SubE80 [Nicotiana tabacum]	defense response
SGN-U583016	TMV response-related gene product [Nicotiana tabacum]	defense response
SGN-U569121	Avr9/Cf-9 rapidly elicited protein 216 [Nicotiana tabacum]	defense response
SGN-U581131	elicitor inducible protein [Nicotiana tabacum]	defense response
SGN-U571708	bacterial-induced peroxidase precursor [Gossypium hirsutum]	defense response
SGN-U566251	bacterial-induced peroxidase precursor [Gossypium hirsutum]	defense response
SGN-U574797	hin1-like protein [Solanum tuberosum]	defense response
SGN-U574800	hin1-like protein [Solanum tuberosum]	defense response
SGN-U574796	hin1-like protein [Solanum tuberosum]	defense response
SGN-U574766	wound-induced protein Sn-1, vacuolar membrane - pepper	defense response
SGN-U577521	wound-induced protein Sn-1, vacuolar membrane - pepper	defense response
SGN-U576244	phosphoenolpyruvate carboxylase 2 [Lycopersicon esculentum]	energy pathways
SGN-U577904	glucose-1-phosphate adenylyltransferase (EC 2.7.7.27) small chain - potato	energy pathways
SGN-U593570	NADH dehydrogenase subunit 7 [Atropa belladonna]	energy pathways
SGN-U581437	auxin growth promotor protein [Lycopersicon esculentum]	hormone responses; auxin responses

SGN-U583983	auxin-responsive protein family [Arabidopsis thaliana]	hormone responses; auxin responses
SGN-U577682	aux/IAA protein [Populus tremula x Populus tremuloides]	hormone responses; auxin responses
SGN-U577967	auxin-induced SAUR-like protein [Capsicum annuum]	hormone responses; auxin responses
SGN-U582094	auxin-repressed protein [Prunus armeniaca]	hormone responses; auxin responses
SGN-U579545	Pathogenesis-related leaf protein 6 precursor (P6) (Ethylene-induced protein P1) (P14) (P14A) (PR protein)	hormone responses; ethylene responses
SGN-U578486	late embryogenesis (Lea)-like protein ER5, ethylene-responsive - tomato	hormone responses; ethylene responses
SGN-U584916	Ethylene-responsive proteinase inhibitor I precursor	hormone responses; ethylene responses
SGN-U564534	Zeatin O-glucosyltransferase (Trans-zeatin O-beta-D-glucosyltransferase)	hormone responses; cytokinin biosynthesis
SGN-U590701	S-adenosylmethionine synthetase 2 (Methionine adenosyltransferase 2) (AdoMet synthetase 2)	hormone responses; ethylene biosynthesis
SGN-U583015	S-adenosylmethionine synthetase 3 (Methionine adenosyltransferase 3) (AdoMet synthetase 3)	hormone responses; ethylene biosynthesis
SGN-U580634	S-adenosylmethionine synthetase 1 (Methionine adenosyltransferase 1) (AdoMet synthetase 1)	hormone responses; ethylene biosynthesis
SGN-U593578	S-adenosylmethionine synthetase 1 (Methionine adenosyltransferase 1) (AdoMet synthetase 1)	hormone responses; ethylene biosynthesis
SGN-U580948	arginine decarboxylase 1 [Datura stramonium]	hormone responses;polyamine biosynthesis;
SGN-U578741	arginine decarboxylase 1 [Datura stramonium]	hormone responses;polyamine biosynthesis;
SGN-U578125	aldolase, plastidic NPALDP1 [Nicotiana paniculata]	photosynthesis; Calvin cycle
SGN-U580944	phosphoribulokinase precursor [Oryza sativa (indica cultivar-group)]	photosynthesis; Calvin cycle
SGN-U580022	aldolase, plastidic NPALDP1 [Nicotiana paniculata]	photosynthesis; Calvin cycle
SGN-U581053	aldolase, plastidic [Nicotiana paniculata]	photosynthesis; Calvin cycle
SGN-U578421	Ribulose bisphosphate carboxylase small chain 3A/3C, chloroplast precursor (RuBisCO small subunit 3A/3C)	photosynthesis; Calvin cycle
SGN-U580869	Ribulose bisphosphate carboxylase small chain 3A/3C, chloroplast precursor (RuBisCO small subunit 3A/3C)	photosynthesis; Calvin cycle
SGN-U577570	Ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor (RuBisCO activase) (RA)	photosynthesis; Calvin cycle
SGN-U580240	Ribulose bisphosphate carboxylase/oxygenase activase 1, chloroplast precursor (RuBisCO activase 1) (RA 1)	photosynthesis; Calvin cycle
SGN-U579099	Chlorophyll A-B binding protein 1B, chloroplast precursor (LHCII type I CAB-1B) (LHCP)	photosynthesis; light reactions
SGN-U579405	Chlorophyll A-B binding protein 1B, chloroplast precursor (LHCII type I CAB-1B) (LHCP)	photosynthesis; light reactions
SGN-U578505	Chlorophyll A-B binding protein 1B, chloroplast precursor (LHCII type I CAB-1B) (LHCP)	photosynthesis; light reactions
SGN-U581203	Chlorophyll A-B binding protein 3C, chloroplast precursor (LHCII type I CAB-3C) (LHCP)	photosynthesis; light reactions
SGN-U579959	Glutamyl-tRNA reductase 1, chloroplast precursor (GluTR)	photosynthesis; chlorophyll biosynthesis
SGN-U564860	asparaginyl-tRNA synthetase (AsnRS) (SYNO) [Arabidopsis thaliana]	protein biosynthesis
SGN-U576007	ribosomal protein S17	protein biosynthesis;
SGN-U581129	ribosomal protein, 40S ribosomal protein S8	protein biosynthesis
SGN-U565541	ribosomal protein S17	protein biosynthesis
SGN-U576538	ribosomal protein, 30S ribosomal protein 3, chloroplast precursor (PSRP-3)	protein biosynthesis
SGN-U577927	ribosomal protein, 60S ribosomal protein L35 [Euphorbia esula]	protein biosynthesis
SGN-U581582	Wound-induced proteinase inhibitor I precursor	protein degredation
SGN-U573510	metalloprotease -related [Arabidopsis thaliana]	protein degredation
SGN-U566446	proteasome regulatory non-ATPase subunit [Oryza sativa (japonica cultivar-group)]	protein degredation
SGN-U572209	E2, ubiquitin-conjugating enzyme UBC7 [Arabidopsis thaliana]	protein degredation

SGN-U577496	ubiquitin-conjugating enzyme family [Arabidopsis thaliana]	protein degredation
SGN-U571577	F-box protein family [Arabidopsis thaliana]	protein degredation
SGN-U592408	Phytoene synthase 1, chloroplast precursor (Fruit ripening specific protein pTOM5)	secondary metabolism; carotenoid biosynthesis
SGN-U580050	Phenylalanine ammonia-lyase	secondary metabolism; phenylpropanoid biosynthesis
SGN-U579042	tyramine hydroxycinnamoyl transferase [Solanum tuberosum]	secondary metabolism; phenylpropanoid biosynthesis
SGN-U579184	tyramine hydroxycinnamoyl transferase [Solanum tuberosum]	secondary metabolism; phenylpropanoid biosynthesis
SGN-U581707	calmodulin, putative [Arabidopsis thaliana]	signal transduction
SGN-U581995	calmodulin [Arabidopsis thaliana]	signal transduction
SGN-U569581	Calcium-binding protein CAST	signal transduction
SGN-U585888	Ca2+-dependent lipid-binding protein [Oryza sativa (japonica cultivar-group)]	signal transduction
SGN-U575004	calcium-binding EF-hand family protein [Arabidopsis thaliana]	signal transduction
SGN-U566692	calcium binding protein [Sesbania rostrata]	signal transduction
SGN-U563913	protein kinase [Arabidopsis thaliana]	signal transduction
SGN-U563330	protein kinase [Oryza sativa]	signal transduction
SGN-U567335	mitogen-activated protein kinase 3 [Lycopersicon esculentum]	signal transduction
SGN-U580172	S-locus protein kinase, putative [Arabidopsis thaliana]	signal transduction
SGN-U572172	Serine/threonine Kinase [Persea americana]	signal transduction
SGN-U595377	calreticulin [Oryza sativa (japonica cultivar-group)]	stress responses
SGN-U600782	DnaJ protein family [Arabidopsis thaliana]	stress responses
SGN-U579132	heat shock protein, small heat shock protein HCT2 [Lycopersicon esculentum]	stress responses
SGN-U579872	dnaK-type molecular chaperone hsc70-3 - tomato	stress responses
SGN-U583160	dehydration-induced protein family [Arabidopsis thaliana]	stress responses
SGN-U578955	MADS-box protein 15 [Petunia x hybrida]	transcription factor
SGN-U563810	DNA-binding protein NtWRKY3 [Nicotiana tabacum]	transcription factor
SGN-U582818	Myb-like protein P [Saussurea medusa]	transcription factor
SGN-U578133	ZPT2-13 [Petunia x hybrida]	transcription factor
SGN-U563809	DNA-binding protein NtWRKY3 [Nicotiana tabacum]	transcription factor
SGN-U580201	PHAP2A protein [Petunia x hybrida]	transcription factor
SGN-U578418	CCCH-type zinc finger protein -related [Arabidopsis thaliana]	transcription factor
SGN-U585671	transcriptional co-activator (KELP) -related [Arabidopsis thaliana]	transcription factor
SGN-U576515	SPF1 protein - sweet potato	transcription factor
SGN-U566778	WIZZ [Nicotiana tabacum]	transcription factor
SGN-U586236	RING protein [Populus x canescens]	transcription factor
SGN-U577434	WRKY family transcription factor [Arabidopsis thaliana]	transcription factor
SGN-U568609	No apical meristem (NAM) protein family [Arabidopsis thaliana]	transcription factor
SGN-U572337	ZPT2-13 [Petunia x hybrida]	transcription factor
SGN-U569474	tuber-specific and sucrose-responsive element binding factor [Solanum tuberosum]	transcription factor
SGN-U566776	WIZZ [Nicotiana tabacum]	transcription factor
SGN-U564952	ethylene responsive element binding factor [Nicotiana tabacum]	transcription factor
SGN-U564955	ethylene response factor 3 [Lycopersicon esculentum]	transcription factor
SGN-U579692	JERF2 [Lycopersicon esculentum]	transcription factor
SGN-U579511	mitochondrial dicarboxylate carrier protein [Arabidopsis	transport

	thaliana]	
SGN-U584494	SEC14 cytosolic factor, putative [Arabidopsis thaliana]	transport
SGN-U584460	lipid transfer protein LTP1 precursor [Capsicum annuum]	transport
SGN-U566668	VAMP protein SEC22 [Arabidopsis thaliana]	transport
SGN-U573568	porin, 36 kDa outer mitochondrial membrane protein porin (Voltage-dependent anion-selective channel protein) (VDAC) (POM 36)	transport
SGN-U566579	ATPase, AAA-type ATPase family [Arabidopsis thaliana]	transport
SGN-U571409	calcium ATPase [Mesembryanthemum crystallinum]	transport
SGN-U581509	ATP synthase CF0 A chain [Nicotiana tabacum]	transport
SGN-U583491	ATP synthase delta chain, chloroplast precursor	transport
SGN-U579050	ABC transporter [Oryza sativa (japonica cultivar-group)]	transport
SGN-U578689	thiazole biosynthetic protein, chloroplast [Nicotiana tabacum]	transport
SGN-U580030	thiazole biosynthetic protein, chloroplast [Nicotiana tabacum]	transport
SGN-U569048	boron transporter [Oryza sativa (japonica cultivar-group)]	transport
SGN-U599308	nitrate transporter NRT1-5 [Glycine max]	transport
SGN-U578370	nucleoside transporter ENT3 -related [Arabidopsis thaliana]	transport
SGN-U585200	unknown protein, sequence homology, OSJNBa0084K20.1	other; unknown protein;
SGN-U583960	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U563903	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U572507	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U573154	unknown protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U601381	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U566580	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U565946	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U567213	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U573301	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U581395	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U579198	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U580513	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U574387	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U563814	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U578180	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U584330	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U577844	unknown protein [Populus tremuloides]	other; unknown protein;
SGN-U565084	unknown protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U566344	unknown protein, sequence homology, At1g17620 [Arabidopsis thaliana]	other; unknown protein;
SGN-U578464	unknown protein, T13J8.30 - Arabidopsis thaliana	other; unknown protein;
SGN-U578016	unknown protein, sequence homology, orf107a [Arabidopsis thaliana]	other; unknown protein;
SGN-U570403	unknown protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U566043	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U569300	unknown protein, T27D20.7 - Arabidopsis thaliana	other; unknown protein;
SGN-U568481	unknown protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U577907	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U581627	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U574899	unknown protein [Arabidopsis thaliana]	other; unknown protein;

SGN-U572810	unknown protein [Euphorbia esula]	other; unknown protein;
SGN-U569177	unknown protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U598004	unknown protein [Danio rerio]	other; unknown protein;
SGN-U570797	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U566851	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U573160	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U566252	unknown protein, T17J13.260 - Arabidopsis thaliana	other; unknown protein;
SGN-U574331	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U580783	unknown protein - Arabidopsis thaliana	other; unknown protein;
SGN-U564822	unknown protein, [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U564872	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U578910	zinc finger protein [Capsicum annuum]	other; zinc finger;

Table 3-S2 Down regulated genes (>2X, p<0,05) *nor* vs *wt* fruits at the BK stage

SGN	***ID/best blast***	***Gene Ontology***
SGN-U569828	branched-chain amino acid aminotransferase [Capsicum annuum]	amino acid metabolism; alanine metabolism;
SGN-U582407	serine acetyltransferase 4 [Nicotiana tabacum]	amino acid metabolism
SGN-U585413	cysteine synthase, cytosolic [Solanum tuberosum]	amino acid metabolism
SGN-U580845	Peptide methionine sulfoxide reductase (Protein-methionine-S-oxide reductase) (Peptide Met(O) reductase) (Fruit-ripening protein E4)	amino acid metabolism
SGN-U576881	tryptophan synthase, alpha subunit (TSA1), putative [Arabidopsis thaliana]	amino acid metabolism
SGN-U578845	Histidine decarboxylase (HDC) (TOM92)	amino acid metabolism
SGN-U580826	Histidine decarboxylase (HDC) (TOM92)	amino acid metabolism
SGN-U577194	ss-galactosidase [Lycopersicon esculentum]	carbohydrate metabolism
SGN-U581969	UDP-glucose dehydrogenase [Oryza sativa (japonica cultivar-group)]	carbohydrate metabolism
SGN-U578195	acid invertase, vacuolar invertase, beta-fructofuranosidase, sucrose-6-phosphate hydrolase	carbohydrate metabolism
SGN-U578305	acid invertase, vacuolar invertase, beta-fructofuranosidase, sucrose-6-phosphate hydrolase	carbohydrate metabolism
SGN-U579395	beta-carbonic anhydrase [Nicotiana tabacum]	carbohydrate metabolism
SGN-U585246	pectate lyase [Malus x domestica]	cell wall
SGN-U585247	pectate lyase [Malus x domestica]	cell wall
SGN-U585252	pectate lyase [Salix gilgiana]	cell wall
SGN-U577423	Polygalacturonase 2A precursor (PG-2A) (Pectinase)	cell wall
SGN-U578473	expansin 1 - tomato	cell wall
SGN-U572461	NP24 protein precursor (Pathogenesis-related protein PR P23) (Salt-induced protein)	defense response
SGN-U573507	resistance protein homolog RGC2a - garden lettuce	defense response
SGN-U579414	NP24 protein precursor (Pathogenesis-related protein PR P23) (Salt-induced protein)	defense response
SGN-U574678	enolase (2-phospho-D-glycerate hydroylase) -related [Arabidopsis thaliana]	energy pathways
SGN-U569271	pyruvate decarboxylase [Solanum tuberosum]	energy pathways
SGN-U604154	aldehyde dehydrogenase 1 precursor [Lotus corniculatus]	energy pathways
SGN-U575484	NADH glutamate synthase isoform 2 [Phaseolus vulgaris]	energy pathways
SGN-U578258	NADH2 dehydrogenase (ubiquinone) (EC 1.6.5.3) chain	energy pathways

	TYKY.2 precursor - potato	
SGN-U579240	growth regulator protein [Arabidopsis thaliana]	hormone responses
SGN-U577938	ACC oxidase homolog (Protein E8)	hormone responses; ethylene responses
SGN-U578448	ACC oxidase homolog (Protein E8)	hormone responses; ethylene responses
SGN-U579236	ACC oxidase homolog (Protein E8)	hormone responses; ethylene responses
SGN-U580403	ACC oxidase homolog (Protein E8)	hormone responses; ethylene responses
SGN-U580508	ACC oxidase homolog (Protein E8)	hormone responses; ethylene responses
SGN-U593546	ACC oxidase homolog (Protein E8)	hormone responses; ethylene responses
SGN-U580538	ethylene receptor - tomato (strain UC82-B)	hormone responses; ethylene responses
SGN-U569393	ethylene responsive element binding factor 2 (EREBP-2) [Arabidopsis thaliana]	hormone responses; ethylene responses
SGN-U577742	Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase) (AdoHcyase) (Cytokinin binding protein CBP57)	hormone responses; cytokinin biosynthesis
SGN-U581636	Adenosylhomocysteinase (S-adenosyl-L-homocysteine hydrolase) (AdoHcyase) (Cytokinin binding protein CBP57)	hormone responses; cytokinin biosynthesis
SGN-U567978	ACC synthase, 1-aminocyclopropane-1-carboxylate synthase 2 (ACC synthase 2) (S-adenosyl-L-methionine methylthioadenosine-lyase 2) (ACS-2)	hormone responses; ethylene biosynthesis
SGN-U578607	ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1) (Ethylene-forming enzyme) (EFE) (Protein pTOM 13)	hormone responses; ethylene biosynthesis
SGN-U578701	ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase 1 (ACC oxidase 1) (Ethylene-forming enzyme) (EFE) (Protein pTOM 13)	hormone responses; ethylene biosynthesis
SGN-U581587	Gibberellin 2-oxidase [Cucurbita maxima]	hormone responses; gibberellin biosynthesis
SGN-U581955	gibberellin 20-oxidase-3; 20ox-3 [Lycopersicon esculentum]	hormone responses; gibberellin biosynthesis
SGN-U565692	polyamine oxidase, putative [Arabidopsis thaliana]	hormone responses; polyamine biosynthesis
SGN-U567681	glucosyltransferase IS5a (EC 2.4.1.-), salicylate-induced - common tobacco	hormone responses; salicylic acid responses
SGN-U574291	phototropic response protein, putative [Arabidopsis thaliana]	light responses
SGN-U581604	early light induced protein [Arachis hypogaea]	light responses
SGN-U563587	DNA photolyase - like protein [Arabidopsis thaliana]	light responses
SGN-U581560	beta-ketoacyl-CoA synthase, putative [Arabidopsis thaliana]	lipid metabolism
SGN-U588810	acetyl Co-A acetyltransferase [Hevea brasiliensis]	lipid metabolism
SGN-U574951	alpha-carboxyltransferase aCT-1 precursor - soybean	lipid metabolism
SGN-U572041	lipoxygenase (EC 1.13.11.12) loxC, chloroplast - tomato	lipid metabolism
SGN-U577893	lipoxygenase [Lycopersicon esculentum]	lipid metabolism
SGN-U578028	lipoxygenase (EC 1.13.11.12) - tomato	lipid metabolism
SGN-U573245	shoot gravitropism 2 [Arabidopsis thaliana]	lipid metabolism
SGN-U579216	RNA-binding protein, putative [Arabidopsis thaliana]	nucleic acid metabolism
SGN-U571526	endonuclease/exonuclease/phosphatase family [Arabidopsis thaliana]	nucleic acid metabolism
SGN-U569088	DNA (cytosine-5-)-methyltransferase (EC 2.1.1.37) - tomato	nucleic acid metabolism
SGN-U581166	cytidine deaminase - like [Arabidopsis thaliana]	nucleic acid metabolism
SGN-U573535	poly(A) polymerase -related [Arabidopsis thaliana]	nucleic acid metabolism
SGN-U589658	pentatricopeptide (PPR) repeat-containing protein [Arabidopsis thaliana]	nucleic acid metabolism
SGN-U580994	Ribulose bisphosphate carboxylase small chain 3A/3C, chloroplast precursor (RuBisCO small subunit 3A/3C)	photosynthesis; Calvin cycle
SGN-U577253	Plastocyanin, chloroplast precursor	photosynthesis; light reactions
SGN-U582904	ferredoxin-NADP+ oxidoreductase, chloroplast [Capsicum annuum]	photosynthesis; light reactions
SGN-U579113	Chlorophyll A-B binding protein 4, chloroplast precursor (LHCII type I CAB-4) (LHCP)	photosynthesis; light reactions

SGN-U580293	chlorophyll a oxygenase (chlorophyll b synthase) [Arabidopsis thaliana]	photosynthesis; pigment biosynthesis
SGN-U586518	isoleucyl-tRNA synthetase [Arabidopsis thaliana]	protein biosynthesis
SGN-U571624	phenylalanyl-trna synthetase - like protein [Arabidopsis thaliana]	protein biosynthesis
SGN-U585039	phenylalanine-tRNA synthetase-related protein [Arabidopsis thaliana]	protein biosynthesis
SGN-U573618	cyclophilin [Oryza sativa (japonica cultivar-group)]	protein biosynthesis
SGN-U579080	ribosomal protein S29 [Oryza sativa (japonica cultivar-group)]	protein biosynthesis
SGN-U592334	Proteinase inhibitor type II CEVI57 precursor	protein degredation
SGN-U568670	proteasome subunit alpha type 4 (20S proteasome alpha subunit C) (20S proteasome subunit alpha-3)	protein degredation
SGN-U568611	beta-carotene hydroxylase [Lycopersicon esculentum]	secondary metabolism; carotenoid biosynthesis
SGN-U578302	Phytoene synthase 2, chloroplast precursor	secondary metabolism; carotenoid biosynthesis
SGN-U580375	phytoene synthase (EC 2.5.1.-) - tomato	secondary metabolism; carotenoid biosynthesis
SGN-U580527	Phytoene synthase 1, chloroplast precursor (Fruit ripening specific protein pTOM5)	secondary metabolism; carotenoid biosynthesis
SGN-U582562	plastid terminal oxidase [Lycopersicon esculentum]	secondary metabolism; carotenoid biosynthesis
SGN-U564570	geranylgeranyl reductase [Nicotiana tabacum]	secondary metabolism; carotenoid biosynthesis
SGN-U573421	geranyl diphosphate synthase (GPPS)(dimethylallyltransferase), putative [Arabidopsis thaliana]	secondary metabolism; carotenoid biosynthesis
SGN-U569057	cinnamyl-alcohol dehydrogenase (EC 1.1.1.195) - apple tree	secondary metabolism; phenylpropanoid biosynthesis
SGN-U571469	malonyl CoA:anthocyanin 5-O-glucoside-6'''-O-malonyltransferase [Perilla frutescens]	secondary metabolism; phenylpropanoid biosynthesis
SGN-U580173	calcium-binding protein (clone Y8) - potato	signal transduction
SGN-U589572	transducin, WD-40 repeat protein family [Arabidopsis thaliana]	signal transduction
SGN-U575600	CBL-interacting protein kinase 1 [Arabidopsis thaliana]	signal transduction
SGN-U577536	protein kinase [Arabidopsis thaliana]	signal transduction
SGN-U568600	Ste-20 related kinase, 3'-partial [Oryza sativa]	signal transduction
SGN-U576547	cyclin-dependent kinase inhibitor [Nicotiana tabacum]	signal transduction
SGN-U564935	leucine-rich repeat receptor-like protein kinase 1 [Populus nigra]	signal transduction
SGN-U585818	leucine-rich repeat transmembrane protein kinase, putative [Arabidopsis thaliana]	signal transduction
SGN-U572172	Serine/threonine Kinase [Persea americana]	signal transduction
SGN-U580176	protein phosphatase 2C (PP2C), putative [Arabidopsis thaliana]	signal transduction
SGN-U593955	protein phosphatase 2C (PP2C) [Fagus sylvatica]	signal transduction
SGN-U593423	calcineurin-like phosphoesterase family [Arabidopsis thaliana]	signal transduction
SGN-U569038	phosphoprotein phosphatase (EC 3.1.3.16) 2A regulatory chain - common tobacco	signal transduction
SGN-U578221	cold-induced glucosyl transferase [Solanum sogarandinum]	stress responses
SGN-U585513	cold acclimation protein WCOR413-like protein [Oryza sativa (japonica cultivar-group)]	stress responses
SGN-U585087	dehydration-induced protein-related [Arabidopsis thaliana]	stress responses
SGN-U562919	heat shock transcription factor 5 (HSF5) [Arabidopsis thaliana]	transcription factor
SGN-U567211	nam-like protein 18 [Petunia x hybrida]	transcription factor
SGN-U568605	nam-like protein 10 [Petunia x hybrida]	transcription factor
SGN-U568610	NACNOR [Lycopersicon esculentum]	transcription factor

SGN-U576158	Transcriptional activator DEMETER (DNA glycosylase-related protein DME)	transcription factor
SGN-U576773	WRKY family transcription factor [Arabidopsis thaliana]	transcription factor
SGN-U577950	TDR4 transcription factor [Lycopersicon esculentum]	transcription factor
SGN-U578128	TDR4 transcription factor [Lycopersicon esculentum]	transcription factor
SGN-U580201	PHAP2A protein [Petunia x hybrida]	transcription factor
SGN-U580800	heat shock transcription factor family [Arabidopsis thaliana]	transcription factor
SGN-U585375	transcriptional factor B3 family [Arabidopsis thaliana]	transcription factor
SGN-U585671	transcriptional co-activator (KELP) -related [Arabidopsis thaliana]	transcription factor
SGN-U593817	homeotic protein VAHOX1 - tomato	transcription factor
SGN-U580868	ATP synthase delta' chain, mitochondrial precursor	transport
SGN-U565599	sorbitol transporter [Prunus cerasus]	transport
SGN-U584113	oligopeptide transporter -related [Arabidopsis thaliana]	transport
SGN-U571497	NTGP4 [Nicotiana tabacum]	other; unknown protein;
SGN-U576432	fibrillarin 2 (AtFib2) [Arabidopsis thaliana]	other; unknown protein;
SGN-U577583	steroleosin [Sesamum indicum]	other; unknown protein;
SGN-U578441	TSI-1 protein - tomato	other; unknown protein;
SGN-U580752	embryo-abundant protein -related [Arabidopsis thaliana]	other; unknown protein;
SGN-U579208	acyltransferase 2 [Capsicum chinense]	other; unknown protein;
SGN-U579261	acyltransferase 2 [Capsicum chinense]	other; unknown protein;
SGN-U577949	Alcohol dehydrogenase 2	other; unknown protein;
SGN-U579420	Alcohol dehydrogenase 2	other; unknown protein;
SGN-U581797	short-chain type alcohol dehydrogenase [Solanum tuberosum]	other; unknown protein;
SGN-U580200	nitropropane dioxygenase [Arabidopsis thaliana]	other; unknown protein;
SGN-U576693	UDP-glycosyltransferase family [Arabidopsis thaliana]	other; unknown protein;
SGN-U578117	phospholipid hydroperoxide glutathione peroxidase (PHGPx)	other; unknown protein;
SGN-U565325	haloacid dehalogenase-like hydrolase family [Arabidopsis thaliana]	other; unknown protein;
SGN-U574333	monooxygenase [Solanum tuberosum]	other; unknown protein;
SGN-U563151	cytochrome P450 86A1 [Arabidopsis thaliana]	other; unknown protein;
SGN-U573731	cytochrome P450-dependent fatty acid hydroxylase [Nicotiana tabacum]	other; unknown protein;
SGN-U574266	Cytochrome P450 71A4 (CYPLXXIA4) (P-450EG2)	other; unknown protein;
SGN-U580108	oxidoreductase, 2OG-Fe(II) oxygenase family [Arabidopsis thaliana]	other; unknown protein;
SGN-U579777	oxidoreductase, zinc-binding dehydrogenase family [Arabidopsis thaliana]	other; unknown protein;
SGN-U577690	peroxidase (EC 1.11.1.7) - common tobacco	other; unknown protein;
SGN-U565828	Fruit-specific protein	other; unknown protein;
SGN-U586128	remorin 1 [Lycopersicon esculentum]	other; unknown protein;
SGN-U567305	proline-rich protein-related [Arabidopsis thaliana]	other; unknown protein;
SGN-U571262	RING finger protein [Cicer arietinum]	other; unknown protein;
SGN-U583316	RING-H2 zinc finger protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U570358	ripening regulated protein DDTFR18 [Lycopersicon esculentum]	other; unknown protein;
SGN-U590493	thaumatin homolog NP24 precursor - tomato	other; unknown protein;
SGN-U590993	unknown	other; unknown protein;
SGN-U598096	unknown	other; unknown protein;
SGN-U563849	unknown protein [Arabidopsis thaliana]	other; unknown protein;

SGN-U564193	unknown protein, sequence homology, [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U565379	unknown protein, 25451-20507 [imported] - Arabidopsis thaliana	other; unknown protein;
SGN-U565729	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U565816	unknown protein [Oryza sativa]	other; unknown protein;
SGN-U566888	unknown protein (protein for MGC:63651) [Danio rerio]	other; unknown protein;
SGN-U566973	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U567163	unknown protein [imported] - Arabidopsis thaliana	other; unknown protein;
SGN-U569129	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U570082	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U572715	unknown protein [Plasmodium yoelii yoelii]	other; unknown protein;
SGN-U573242	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U574010	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U574144	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U574226	unknown protein, sequence homology to 177O13.43 [Solanum bulbocastanum]	other; unknown protein;
SGN-U574534	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U575753	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U577765	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U578119	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U578696	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U578954	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U579081	unknown protein, sequence homology, OSJNBa0093F12.14 [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U579145	unknown protein, [Prunus armeniaca]	other; unknown protein;
SGN-U579170	unknown protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U580274	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U580569	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U580613	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U583807	unknown protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U583856	unknown protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U584569	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U585313	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U586340	unknown protein [Oryza sativa (japonica cultivar-group)]	other; unknown protein;
SGN-U595844	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U598389	unknown protein [Arabidopsis thaliana]	other; unknown protein;
SGN-U568588	no hits found	other; unknown protein;
SGN-U570571	no hits found	other; unknown protein;
SGN-U570655	no hits found	other; unknown protein;
SGN-U572183	no hits found	other; unknown protein;
SGN-U578621	no hits found	other; unknown protein;
SGN-U579305	no hits found	other; unknown protein;
SGN-U580782	no hits found	other; unknown protein;
SGN-U582514	no hits found	other; unknown protein;
SGN-U582765	no hits found	other; unknown protein;
SGN-U585166	no hits found	other; unknown protein;
SGN-U585199	no hits found	other; unknown protein;
SGN-U601903	no hits found	other; unknown protein;
SGN-U567697	zinc finger protein [Arabidopsis thaliana]	other; unknown protein;

CHAPTER 4 DEVELOPMENT OFAN INDUCIBLE SYSTEM FOR CONTROLLED EXPRESSION OF RIN AND NOR IN TOMATO FRUITS

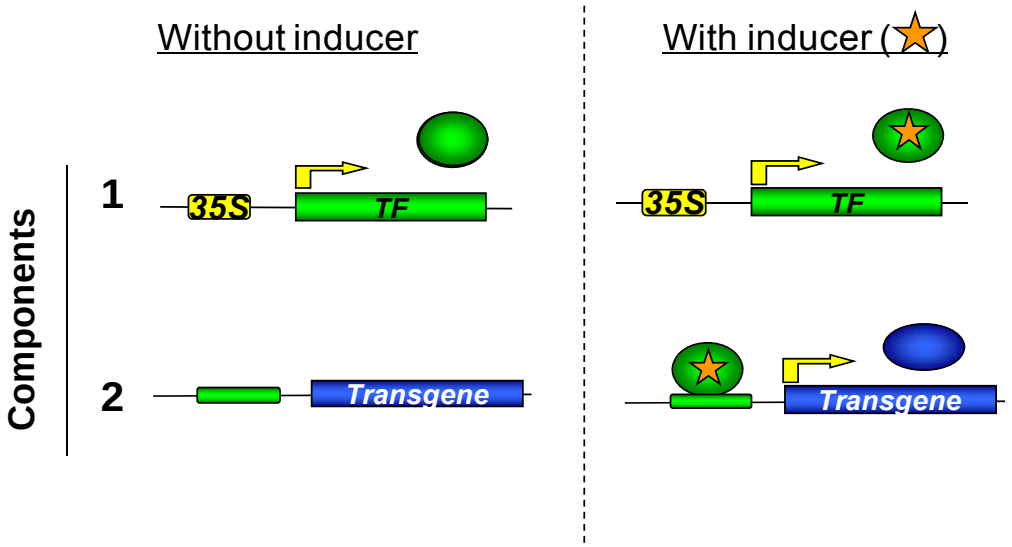
4.1 INTRODUCTION

Tomato fruit ripening is a complex process requiring the precise temporal control of distinct biochemical pathways (Seymour, 1993), however the regulatory mechanisms involved in initiating and coordinating those different pathways are only partially understood. Studies of the tomato ripening defective single locus mutants *rin* and *nor* have led to the identification of two important transcription factors involved in the ripening. The *RIN* gene has been shown to encode a MADS box transcription factor (Vrebalov et al., 2002), and the *NOR* gene a NAC domain transcription factor (Vrebalov, pers. comm.). Molecular characterization of these proteins revealed that they are expressed in mature fruits slightly before the initiation of the ripening program. Transcriptomic studies performed on the *rin* and *nor* mutants (see Chapter 2 and 3) have shed some light on the numerous biochemical pathways, including primary metabolism, ethylene synthesis, cell wall degradation and carotenoid accumulation, that are influenced by RIN and NOR. These studies provide a general a picture of the overall impacts of misexpression of these two key transcription factors. Questions however remain concerning the specific mechanism by which the RIN and NOR proteins function to influence the regulation of these biochemical pathways. To better understand the transcriptional cascade involved in fruit ripening, an important goal is to identify the genes that are directly regulated by RIN and NOR. In order to address this question, I created an inducible system allowing the temporal control of expression of *RIN* and *NOR*. The objective was to precisely control their respective activities while minimizing any background that would result from normal expression

of the endogenous genes by introducing inducible versions of *RIN* and *NOR* into their respective mutant backgrounds (*rin* and *nor*). A transcriptomic survey would then allow the identification of genes whose expression rapidly changes following the induction of *RIN* or *NOR* transgenes. These genes would likely represent primary regulatory targets of the RIN and NOR proteins.

I tested two different systems to regulate the expression of the *RIN* and *NOR* genes in tomato fruits. Both system selected are based on a two-component system to provide inducibility (see Figure 4-1). In each case, the first component consists of a transgene containing the gene of interest (*RIN* or *NOR)* under the control of an inducible promoter. This promoter contains specific *cis*-elements that can recruit the second component of the system, a transcription factor. The expression of this transcription factor is regulated by the CaMV35S promoter and is thus constitutive. The inducibility of the system relies on the fact that, although constantly expressed, the transcription factor is unable to bind its target DNA in the absence of the inducer. The addition of the inducer to the system allows binding of the transcription factor to its target promoter and transcription of the transgene of interest.

One system tested was the ethanol inducible AlcR system based on the ethanol regulon of the ascomycetes fungus *Aspergillus nidalans* (Caddick et al., 1998). The constitutively expressed AlcR transcription factor is recruited to the promoter of the alcohol dehydrogenase *AlcA* gene only when ethanol is present. This system has been successfully used in several species including *N. tabaccum*, *A. thaliana*, *S. tuberosum* (potatoes), *Brassica napus* and *S. lycopersicum* (Caddick et al., 1998; Roslan et al., 2001; Sweetman et al., 2002; Garoosi et al., 2005). Garoosi et al. (2005) examined the functionality of the AlcR ethanol induction system in mature tomato leaves (cv *Ailsa Craig*) but did not discuss the effectiveness of the system in fruits (Garoosi et al., 2005).



System	Inducible TF	Promoter	Inducer
AlcR	AlcR	*AlcA* (*A.nidulans*)	Ethanol
Glucocorticoid	lac repressor - Gal4AD-GR LBD	*lac* (*E. coli*)	glucocorticoid

Figure 4-1 Scheme of the inducible system used for the generation of RIN and NOR inducible lines. Two components are required for induction. The first is a constitutively-expressed (driven by CAM35S promoter) transcription factor (TF), the second is a vector containing the transgene of interest under the control of a promoter specifically bound by the TF. The TF can only bind and induce expression of the transgene when bound to the inducer. The components of the two systems used in this experiment are listed below.

In the glucocorticoid system (Craft et al., 2005; Samalova et al., 2005), the transcription factor component is a chimera of the *E. coli* lac repressor DNA binding protein, the *S. cerevisiae* Gal4 activation domain and the ligand binding region (LBR) of the rat glucocorticoid receptor (GR). This chimera is recruited to a *lac* promoter located upstream of the inducible transgene only when a glucocorticoid, such as dexamethasone, is applied to the system. This system has been reported to work

efficiently in *A. thaliana* and *N. tabaccum*; however, at the time I started this study, no report had yet demonstrated the functionality of this system in tomato plants. Recently, this system was successfully used to induce the expression of the mutant *A.thaliana* ethylene receptor *etr1-1* in tomato *cv Microtom* fruits (Gallie, 2010).

4.2 MATERIAL AND METHODS

Plant inducer treatment

Plants (cv. Ac) were grown under greenhouse conditions until the two first trusses produced BK stage fruits. Induction was attempted by watering the roots with 200ml of a 1% ethanol solution (for the AlcR system) or 50µM dexamethasone (Sigma) solution (glucocorticoid system). This treatment was repeated daily for up to 2 weeks. Detached shoots and trusses bearing fruits were induced by incubation in the inducer solution for the period of the experiment. The pericarp of detached fruit was injected with water or a 5µM DEX solution using a 1ml syringe and 30G1/2 inch needle (Becton Dickinson, NJ). For the sliced fruit induction, BK fruits were collected, cut with a razor blade into 2-5mm thick slices, placed in a petri dish containing a filter paper imbibed with 50µM of DEX and incubated at room temperature for the duration of the induction.

Consrtucts and plant transformation

The pJRF construct was obtained as follows: *RIN* cDNA was PCR amplified using the primers RIN1-*Kpn*/*SalI* F: 5'-TATAGGTACCGTCGACATGGGTAGAGGGAAA-GTAG-3' and RIN1 BamHI-SalI : TATAGTCGACGGATCCAAGCATCCATCCA-G-3', digested with *SalI*, and cloned into the *SalI* site of the pACN vector to generate pACN-RIN. A double stranded oligonucleotide coding for the FLAG tag was made by annealing the following primers : FLAG BHI F: 5'-GATCCGACTACAAGGACG-ACGATGACAAATAAG-3' and FLAG BHI R : 5'-GATCCTTATTTGTCATCG-

TCGTCCTTGTAGTCG-3'. This oligonucleotide was then inserted in the BamHI site of pACN-RIN to generate pACN-RIN-FLAG. The primers AlcR F (5'-TATAGTTC-CGACCTAGGATTGGATG-3') and pACN R (5'-TATACCCGGGATCATCGCAA-GACCG-3') were used to amplify the AlcR-RIN-FLAG region of the pACN-RIN-FLAG vector, and were then digested with *AvrII* and *SmaI* and cloned into the pJH0022 vector pre-digested with *AvrII* and *ScaI* restriction enzymes.

The pVNOR construct was obtained by PCR amplifying the NOR cDNA using the primers NOR-*KpnI*-*SalI* F (5'-TATAGGTACCGTCGACATGGAAAGTACGGA-TTC-3') and NOR-*BamHI*-*SalI* R (5'-TATAGTCGACGGATCCAGAGTACCA-ATTC-3'). The PCR resulting product was digested with *SalI* and *BamHI* restriction enzymes and cloned into the pVTOP vector pre-digested with the same enzymes to generate the pVNOR construct. The FLAG tag double stranded oligonucleotide was inserted into the *BamHI* site of the pVNOR site, thus generating the pVNF construct.

Western blot analysis

Western blot analysis was performed as described in Chapter 3 using the NOR specific antibody (1:1,000 dilution) or the anti-FLAG antibody (Sigma).

qRT-PCR

qRT-PCR was performed as described in Chapter 2. Gene-specific primers were used to amplify NOR (F: 5'-GCACCACCAATGGATGTGGTTCTT-3', R: 5'-GTAGGC-TTATTCGAATCTCTTCGC-3') and GRTF (F: 5'-CCACTGCAGGAGTCTCACAA-3', R: 5'-AACACCTCGGGTTCAATCAC-3')

GUS staining

GUS staining was performed by incubating fruit slices in staining solution (100mM $NaPO_4$ pH8, 10mM EDTA, 0.2% Triton X-100, 0.1mM $K_3Fe(CN)_6$, 0.1mM $K_4Fe(CN)_6$, 20% methanol, 0.5mM X-gluc) for 16h at 37ºC. Stained fruits were then

put in fixing solution (45% ethanol, 5% formalin, 5% acetic acid) for 16h at 4ºC, and destained with 100% ethanol for 16h at 37ºC.

Microarray analysis

cDNA was derived from RNA isolated from frozen tissue and labeled with cy3 and cy5 dyes and hybridized to the TOM2 oligoarray chip, as described (Alba et al., 2005). Acquisition and analysis of the results was performed as described previously (Fei et al., 2006).

4.3 RESULTS

4.3.1 Ethanol inducible *RIN* expression

The ethanol-inducible system was used to control the expression of a RIN protein containing a C-terminal FLAG tag. The *AlcR::RIN-FLAG* construct was transformed into *rin* to yield the pJRF lines and of the 8 transformed lines recovered, four were selfed to obtain homozygous transgene inserts and used for further analysis. Plants were grown without inducer until maturity under normal greenhouse conditions. No significant vegetative differences between transgenic and non-transgenic plants were observed. Interestingly, the fruits of one transgenic line, pJRF5, showed distinct signs of ripening, in that while they did not ripen entirely, they turned orange and softened significantly more than non-transgenic *rin* fruits (Figure 4-2A). This suggests that the pJRF5 line is leaky and possibly expresses the RIN transgene in the absence of inducer. Surprisingly, no RIN protein could be detected in these fruits when Western blot analysis was performed (Figure 4.2B).

Two explanations can be proposed to explain these seemingly contradictory results. First, it is possible that the *RIN* transgene is only expressed at a level resulting in RIN protein accumulation that is below the detection limit of our Western analysis, but still

enough to trigger a ripening phenotype. Alternatively, the *RIN* transgene could have been expressed transiently during fruit development and I did not perform a Western blot at the appropriate stage to detect the protein. Results reported in chapter 2 suggest that RIN protein accumulation is strongly correlated with transcript levels, which would be consistent with this interpretation. Regardless of the mechanism responsible for this ripening phenotype, this line was not used for subsequent analysis due to the high level of apparent RIN activity in the absence of inducer.

Three other lines were selected for induction assay analysis and plants bearing at least 2 trusses of mature green fruits were watered daily with a 1% ethanol solution. Following a week of this treatment, none of the fruits showed signs of ripening. However, the ethanol treatment had deleterious effects on the plants, as all showed signs of stress, including leaf senescence and anthocyanin accumulation. A reduction in ethanol concentration delayed these toxic side effects but also failed to induce ripening in the transgenic lines. In light of these results, I did not pursue the use of the ethanol inducible system to control the expression of the *NOR* gene and instead focused efforts on the DEX inducible system described below.

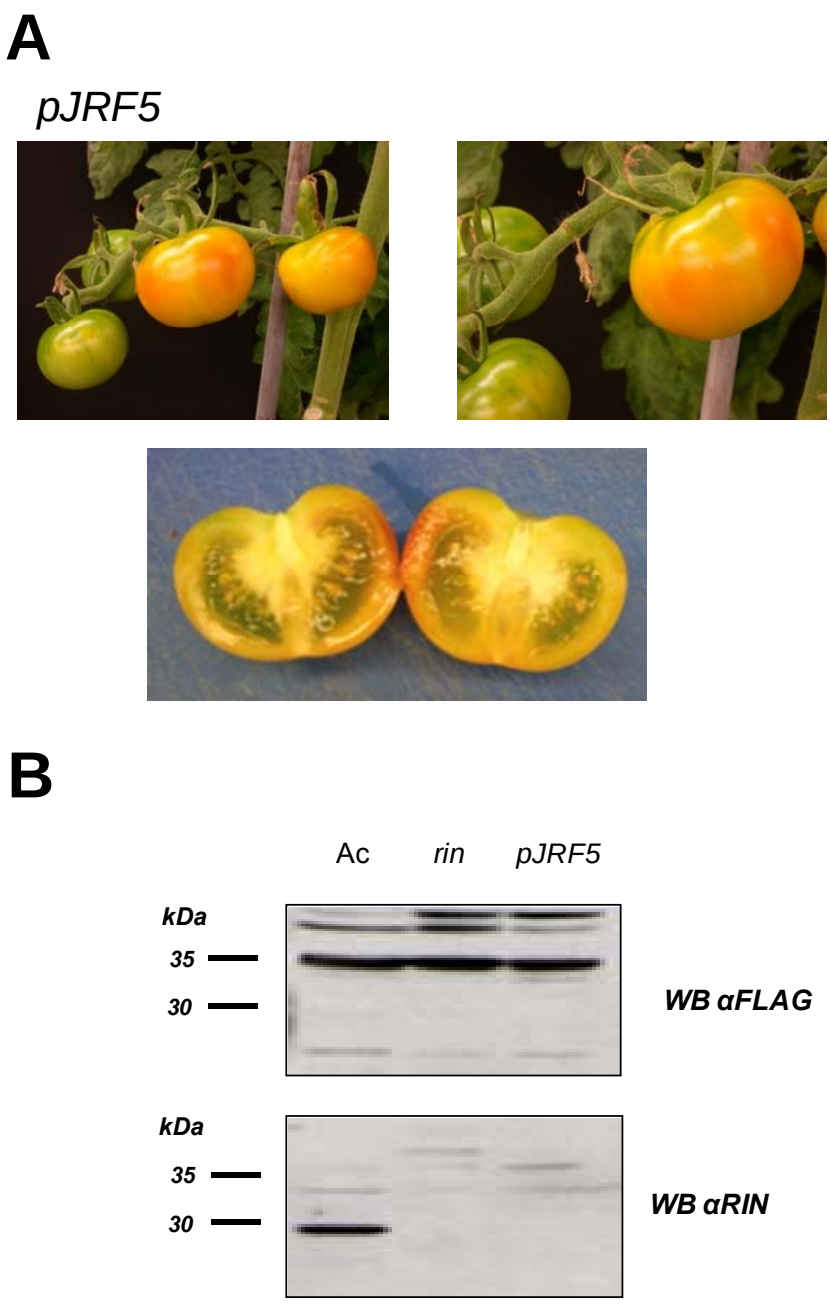


Figure 4-2 Complementation of the *rin* mutation in a single transgeninc inducible RIN line A) Fruits of the transgenic line pJRF5 show signs of ripening. Phenotype is observed in the absence of induction B) Western blot analysis of protein extracted from wt (Ac), *rin* and pJRF5 BK fruits. Top panel : anti- FLAG antibody, bottom panel : anti-RIN antibody.

4.3.2 DEX Inducible system

For the purpose of this experiment, a C-terminally flag-tagged version of the *NOR* cDNA was cloned downstream of the *lac* promoter. The two components of the system (35S:GRTF and *lac*::*NOR-FLAG* vector) were independently transformed into *nor* plants and then crossed to obtain the transgenic lines pVNF containing both transgenes. Several pVNF lines were grown to maturity in the absence of inducer and no abnormal phenotypes were observed in the transgenic plants, indicating that the transgenes and the constitutive expression of GRTF had minimal effects on plant physiology. In order to test the functionality of this system, young shoots from three pVNF lines were harvested and incubated in an aqueous solution containing 50µM of dexamethasone for 24 hours. The abundance of *NOR* and *GRTF* mRNA was measured by qRT-PCR. Figure 4.-3A shows that *NOR* transcription is efficiently activated as early as 6 hours following the addition of the inducer in all of the pVNF lines tested. As expected, the expression level of the GR transcription factor does not correlate with the induction period. I next verified the presence of the NOR protein in the induced leaf tissue by Western blot analysis using the NOR antibody described in Chapter 3. The NOR protein was not detected in the induced leaf tissue (Figure 4-3B). Post-transcriptional mechanisms (miRNA, proteosome degradation) are known to regulate the expression of other NAC proteins (Olsen et al., 2005). It is possible that a similar mechanism limits NOR protein accumulation in the leaves. In order to determine whether NOR protein could accumulate in fruits, I repeated the induction experiment using pVNF trusses bearing mature tomato fruits. I incubated a total of 12 trusses (four per line), each bearing between two to four mature green (MG) fruits, in a solution containing 50µM of dexamethasone for up to 15days. Only one fruit turned slightly orange after being incubated in DEX-containing solution for more than 10 days. Protein extraction and Western blot analysis indicate that this fruit indeed

accumulated NOR protein (Figure 4-3C). The failure of the majority of the fruits to ripen or express NOR protein might be due to the limited amount of inducer reaching the fruit using the protocol used. Indeed, unlike leaf tissue, water uptake into mature fruit is low (Ehret and Ho, 1986, 1986; Ho et al., 1987; Leonardi et al., 1999). However, this result was sufficient to verify that NOR protein could be induced in fruits.

I next focused on method to more efficiently deliver the inducer into the fruit tissue. I watered entire plants with a solution containing DEX, but this failed to induce ripening; extended watering with the DEX solution (> four days) had deleterious effects on plant physiology including senescence of the leaves and causing the subsequent death of the plant. I also directly injected DEX into the pedicule, but this failed to induce ripening and so induction in detached fruits was attempted. Fruits were collected around the BK stage, sliced and incubated on filter paper soaked with 50μM of dexamethasone for up to 24 hours. Inducible expression was monitored by GUS staining, which takes advantage of the presence of an inducible GUS reporter gene present on the same vector as the *NORFLAG* transgene (Craft et al., 2005; Samalova et al., 2005). Figure 4.4A shows the GUS staining of fruit slices incubated in the presence of the inducer. Induction can be detected as early as 6h following the treatment. Western blot analysis was next performed to confirm the presence of the NOR protein in induced fruit slices. As shown on Figure 4-4B, NOR protein was detectable and accumulates in induced fruit around 12h post induction. However, the inductions of GUS and NOR proteins was also detected when the sliced fruits were incubated in the absence of inducer, suggesting that wounding is sufficient to trigger transgene expression. A direct injection approach was also tried in which water or a 5μM DEX solution was directly injected into the pericarp of BK-staged fruits.

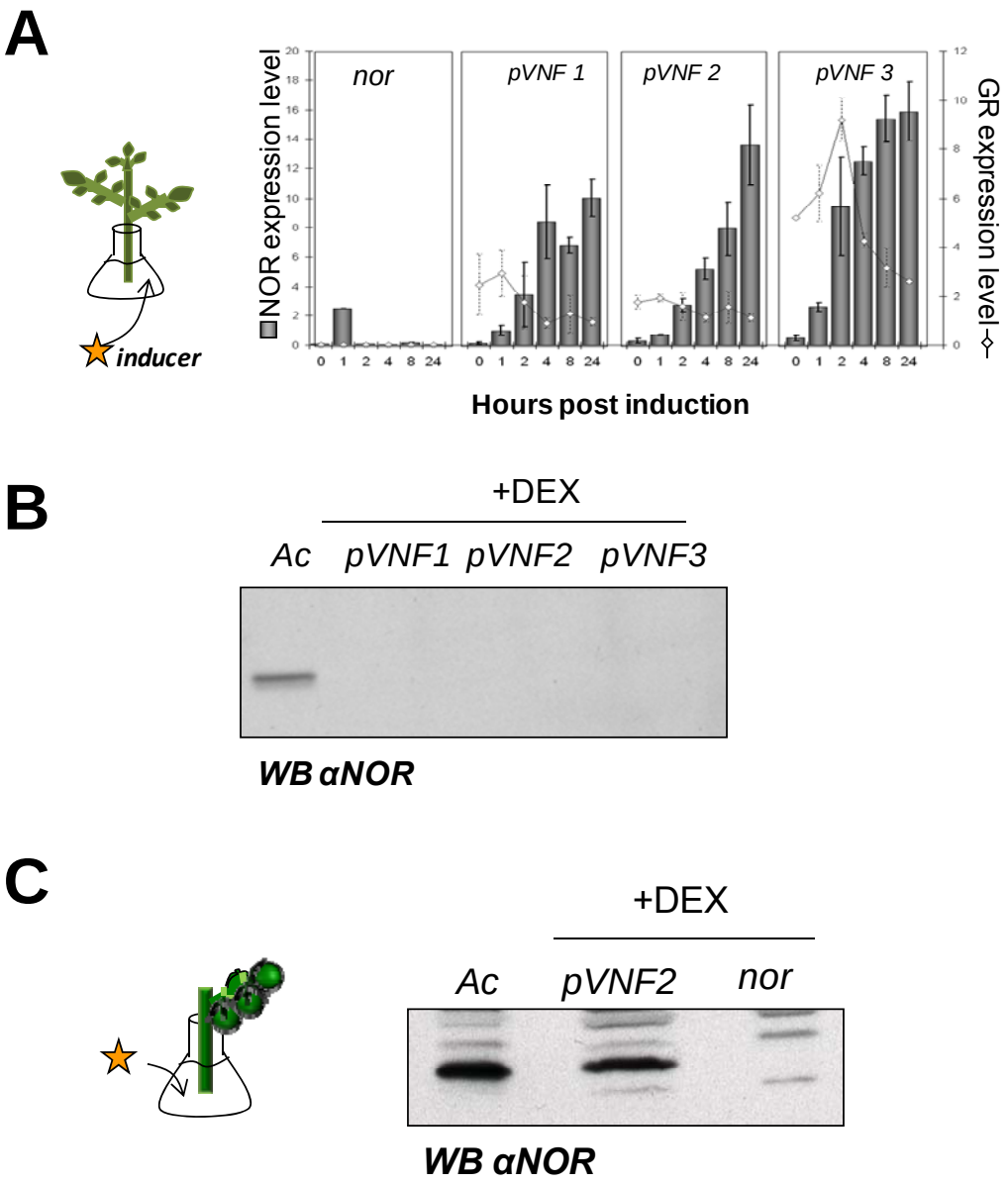


Figure 4-3 Inducible NOR construct induction and protein accumulation. DEX induction assay. Shoots of transgenic lines pVNF-1, -2 and -3, and non transgenic *nor* were cut and incubated in a solution containing 50uM of inducer (yellow star) for up to 24h. qRT-PCR was used to quantify the level of transcription of the NOR transcript (gray bars) and the GRTF (white diamond) in leaves collected on the induced shoots at different time points (x axis) . qRT-PCR values are normalized against 18S. B) NOR Western blot analysis of protein extracted from *nor*, pVNF1/2/ 3 induced leaves after 24 hours in induction solution. C) Trusses of transgenic lines bearing mature fruits were incubated in 50uM DEX for up to 15 days. NOR Western blot analysis of the only fruit (pVNF2 truss) showing signs of ripening after 10 days in inducer solution.

This treatment resulted in some cracking of the fruit. The fruit tissue was collected after 16 hours post injection and induction was monitored by Western blot analysis. As shown in Figure 4-4C, both water and DEX injection led to the expression of the *NOR* transgene, but again, wounding seemed sufficient for induction. I decided to proceed with transcriptomic analysis using the sliced fruit (wounding) induction approach since it produced a more uniform induction than injection (data not shown). A similar approach was used to express a *RIN-FLAG* transgene in the *rin* mutant. pVRF lines containing the GRTF and the RIN FLAG transgene were subjected to an induction assay in both detached shoots and whole plants. No significant increase in transcript level or ripening phenotype was detected so these lines were not further characterized.

4.3.3 Microarray analysis

I collected BK-staged fruit from *nor* and *pVNF* lines, sliced them, and incubated them for 16 hours on filter paper containing 50μM of DEX. Following induction, the fruit slices were frozen in liquid nitrogen, ground, and RNA extracted. A microarray analysis was performed using the TOM2 oligoarray (Fei et al., 2006). It is well known that wounding has a major impact on gene expression, so I therefore wanted to make sure that the genes identified in the microarray experiment were the result of the *NOR* induction and not a result of the wounding response. In order to eliminate wounding response background, I compared the transcriptomic profile of the pVNF-induced line to that of induced (sliced/wounded) *nor* tissue.

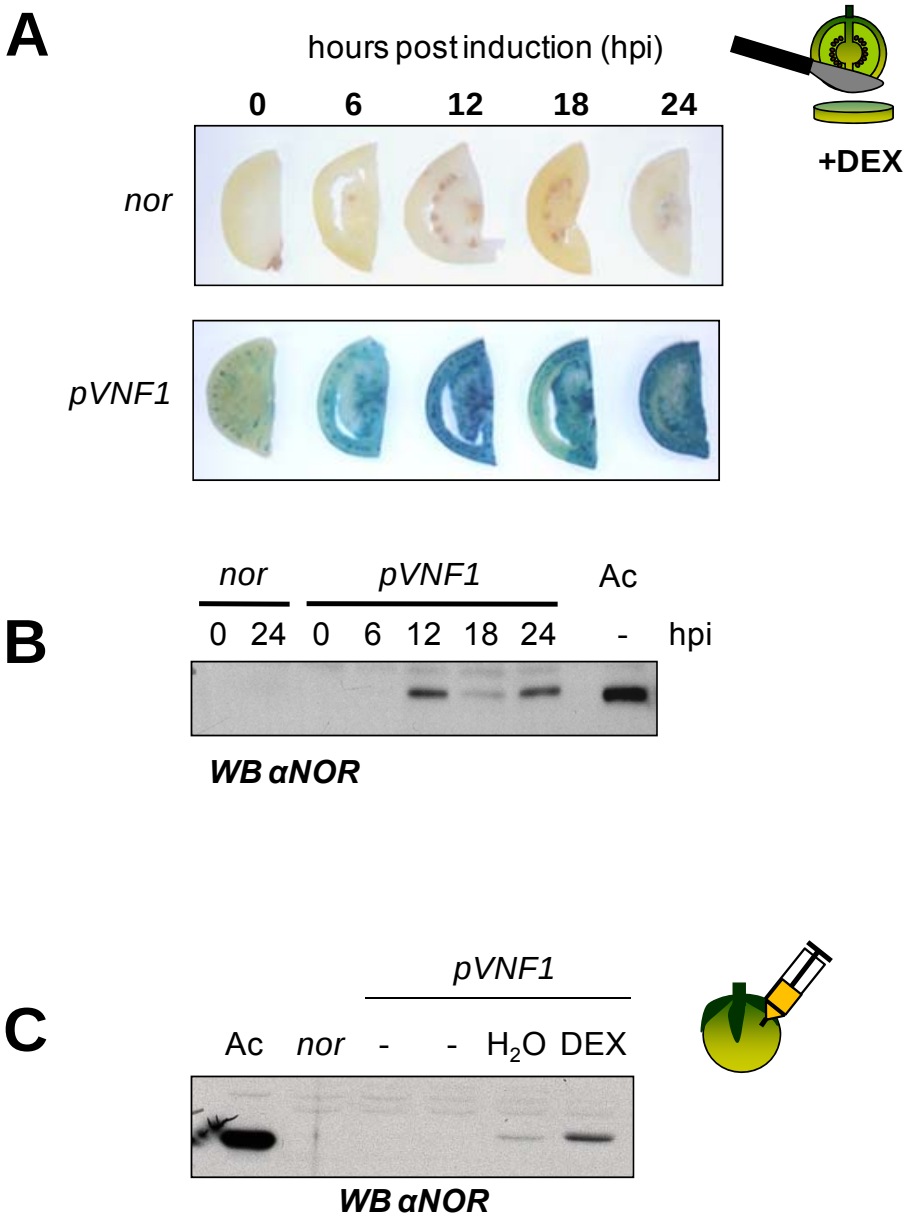


Figure 4-4 Induction in fruit A,B) *nor* and *pVNF1* BK fruits were sliced and incubated on filter paper containing 50µM DEX for up to 24 hours. Fruit slices were collected at different time point and A) stained for GUS activity or B) used for Western blot analysis using the NOR antibody. C) *nor* and *pVNF1* BK fruits were injected with 5µM DEX solution. Tissue was collected after 16 hours and used for Western blot analysis using the NOR antibody.

Genes that are selectively expressed in response to *NOR* induction should be detected

only in pVNF induced fruit slices and not in non-transgenic *nor* slices. Figure 4-5 illustrates the experimental design for this experiment. Four biological replicates were used for the microarray comparison. Genes whose expression is specifically modified by *NOR* were identified by comparing expression in comparison number 1 (pVNOR induced vs. *nor* non induced) and comparison number 2 (*nor* sliced vs *nor* non induced). A total of 125 features showed a twofold or greater increase in expression in the *pVNF* induced lines when compared to the wounding control; however, only four were statistically significant with a p-value below 0.05. Three of these features correspond to the GUS gene. The *CER1* gene was the only tomato gene significantly overexpressed following *NOR*

4.3.4 Microarray analysis

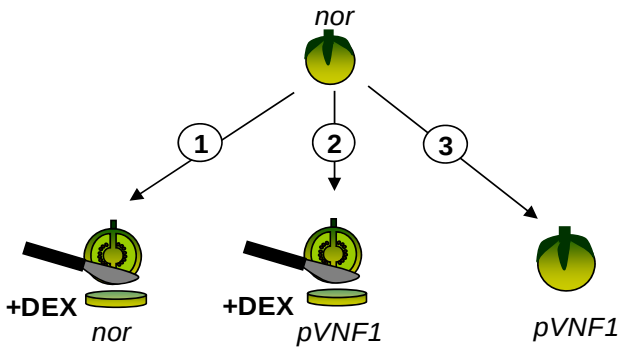
I collected BK-staged fruit from *nor* and *pVNF* lines, sliced them, and incubated them for 16 hours on filter paper containing 50μM of DEX. Following induction, the fruit slices were frozen in liquid nitrogen, ground, and RNA extracted. A microarray analysis was performed using the TOM2 oligoarray (Fei et al., 2006). It is well known that wounding has a major impact on gene expression, so I therefore wanted to make sure that the genes identified in the microarray experiment were the result of the *NOR* induction and not a result of the wounding response. In order to eliminate wounding response background, I compared the transcriptomic profile of the pVNF-induced line to that of induced (sliced/wounded) *nor* tissue. Genes that are selectively expressed in response to *NOR* induction should be detected only in pVNF induced fruit slices and not in non-transgenic *nor* slices. Figure 4-5 illustrates the experimental design for this experiment. Four biological replicates were used for the microarray comparison. Genes whose expression is specifically modified by *NOR* were identified by comparing expression in comparison number 1 (pVNOR induced vs. *nor* non induced)

and comparison number 2 (*nor* sliced vs *nor* non induced). A total of 125 features showed a twofold or greater increase in expression in the *pVNF* induced lines when compared to the wounding control; however, only four were statistically significant with a p-value below 0.05. Three of these features correspond to the GUS gene. The *CER1* gene was the only tomato gene significantly overexpressed following *NOR* expression. This gene encodes an octadecanal decarbonylase involved in epicuticular wax biosynthesis in *A. thaliana* , *O. sativa*, and *Z. mays* (Aarts et al., 1995; Hansen et al., 1997; Islam et al., 2009). Its role in tomato has not been confirmed, but clear changes in the cuticle of *rin* and *nor* tomato fruit have been reported (Kosma et al., 2009). The significance of this result has not been further investigated though this may be a useful gene promoter to target in future ChIP analyses.

4.4 DISCUSSION

The *RIN* and *NOR* genes have been shown to play essential roles in the regulation of fruit ripening. I have shown previously that the expression of numerous genes is influenced by the activity of these two proteins (Chapters 2 and 3). Using a candidate gene approach, I have identified some of the primary targets of RIN and NOR; however, the exact mechanism by which they influence transcription of most of their target genes remains unknown. In order to better understand their function, a valuable approach is the identification of other primary targets. The current project was aimed at generating a high throughput method to identify those primary targets. I used transcriptomic profiling to identify genes whose expression is rapidly changed following the induced expression of *RIN* and *NOR*. Unfortunately, I failed to obtain any significant results with the transgenic approaches employed. The following discussion is primarily aimed at understanding the reasons that could explain this negative result.

A



B

Gene	Comp.1	Comp. 2	2/1	pvalue
GUS	1.2	19.5	16.0	0.02
CER1	1.9	11.9	6.2	0.04
NOR	2.9	6.5	2.2	0.6
RIN	3.8	8.5	2.2	0.6
ACS4	2.0	4.1	2.1	0.8
E8	2.1	4.3	2.0	0.7
Exp	7.2	11.4	1.6	0.9
TDR4	0.9	1.0	1.1	0.9
E4	9.8	9.5	1.0	0.9
NR	2.6	1.9	0.7	0.7
PG2a	1.6	1.3	0.8	0.8

Figure 4-5 Wound induction strategy and array results summary. A) Scheme of the microarray comparison. Comparison 1 : Intact *nor* BK fruit vs induced *nor* BK fruit. Comparison 2 : Intact *nor* BK fruit vs induced *pVNF1* BK fruit. Comparison 3 : : Intact *nor* BK fruit vs intact *pVNF1* BK fruit. 4 biological replicates were used in each comparison. B) Microarray value of ripening-related genes in comparison 1 and 2. Level of NOR specific induction is obtained by dividing value of comp.2 by value of comp.1. p-value of the ratio comp2 vs comp1 is listed in last column. Green: significantly ($p<0.05$) upregulated (fold >2X) genes between comp 1 and 2. Yellow: non-significantly ($p>0.05$) upregulated (fold >2X) genes between comp 1 and 2. Red : gene showing similar expression levels in comp.1 vs comp.2

4.4.1 Non-functional protein

A simple explanation for our negative results is the possibility that the addition of a C-terminal FLAG tag to the RIN and NOR protein negatively influenced their function. Such effect of tags have been reported previously (de Folter et al., 2007), however the fact that a leaky pJRF line showed signs of ripening suggests that the RIN-FLAG protein retains some functionality. Similarly, the fruit of pVNF2 did ripened upon long incubation in the induction solution. Recent data suggest that the addition of a N-terminal tag (GFP) to both RIN and NOR interferes with their function (S. Zhong, pers. comm.) and so it is possible that the FLAG tag reduced the functionality of the RIN and NOR proteins, although some activity must have been retained to explain the observed ripening phenotypes. Alternatively, the single lines that demonstrated some activity may have resulted from mutation in the FLAG sequence or its incomplete transfer in the respective transgenic plants. These insertions in these lines were not analyzed by sequencing to address this possibility.

4.4.2 Inducer delivery

For the inducible systems to work properly, the efficient delivery of the inducer to its target tissue is critical and I encountered some problems in delivering the inducer into mature tomato fruits. For both the DEX and ethanol induction systems, watering the mature plants did not result in significant activation of gene expression in the fruit as monitored by the appearance of the ripening phenotype. This result can be explained in several ways. First, it might be due to the low amount of water (and inducer) imported to the fruit at the time of induction. Fruit development is characterized by three major phases (cell division, cell expansion and ripening) (Gillaspy et al., 1993) and the fruit act as a major sink during the second phase during which water uptake

accounts for almost 90% of the increase in volume during expansion (Ho et al., 1987), but water import is minimal after this stage. Watering the roots when the fruit has reached maturity (MG stage) might not have allowed enough inducer to enter the fruits. Similarly, direct injection of the inducer through the pedicel might be ineffective at that stage as the fruit stops importing nutrients and water. A second potential mechanism relates to the efficient delivery of the inducer from the roots to the fruit. There is a debate as to which vascular elements are responsible for fruit water uptake: numerous studies suggest that phloem is the main contributor (Ho et al., 1987; Adams-Phillips et al., 2004; Plaut et al., 2004; Guichard et al., 2005), whereas other more recent studies argue that the majority of the water uptake is mediated by the xylem (Van Ieperen et al., 2003; Windt et al., 2009). The watering approach would only be effective if water is imported through the xylem. An alternative approach, such as spraying upper leaves with the inducer, could be attempted to try to deliver the inducer through the phloem.

The two hypotheses above could explain why the watering method did not allow effective induction of the fruit. The ethanol induction studies performed in tomato by (Garoosi et al., 2005) did not discuss the efficacy of the system in fruit and it is possible that the authors also experienced difficulties with fruit induction. Concerning the DEX system, a recent study shows the feasibility of fruit induction through root watering (Gallie, 2010). In this paper, the author used the DEX system to induce the expression of a mutant ethylene receptor in tomato fruit and successfully induced gene expression in maturing fruit by watering them. A potentially crucial difference between that assay and our work is the cultivar used to perform the experiment. Gallie (2010) used the dwarf cultivar Microtom, whereas our transgenics were made in Ailsa Craig (Ac). It is possible that the small size and reduced biomass of the microtom tomato plant and fruit allowed transport of sufficient inducer to the fruit tissue for

efficient induction. The Ailsa Craig cultivar used in our study reaches about 1 to 2 meters at the time the first fruits attain the MG stage. Consequently the amount of inducer actually reaching the tissue by the watering method might not be sufficient for induction.

4.4.3 Microarray results

In addition to possible non-functionality of the tagged NOR protein, there are other explanation for our inability to detect any differently expressed genes following NOR induction. First, it is possible that the wounding response masked *NOR*-specific gene regulation. For example, an important component of the wounding response is the production of ethylene (Leon et al., 2001). Considering the critical role played by ethylene in climacteric fruit ripening, it is not unexpected that wounding influences the expression of a number of genes associated with ripening. The *E4* gene is highly sensitive to ethylene, and I detected an increase in *E4* transcript levels in the wounded *nor* tissue (Figure 4-5). The *NOR* gene itself also shows increased expression in both the *nor* and *pVNF* tissue upon wounding and since *NOR* expression is believed to increase in response to ethylene (J. Vrebalov, pers. comm.) this increase in expression is not unexpected. A survey of the literature indicates that many NAC genes are involved in stress responses and it is therefore possible that a certain degree of overlap exists between NOR-regulated genes and wound-induced genes. Mechanical wounding, insect feeding and cold temperature have all been shown to induce the expression of eight NAC genes in *Brassica napus*, five of which have been previously characterized for their role in SAM development (Hegedus et al., 2003). A similar increase in *NAC* genes transcription has also been observed in *A. thaliana*, *O. sativa* and *C. annuum* following stresses such as drought, high salinity and pathogen attack (Tran et al., 2004; Oh et al., 2005; Ohnishi et al., 2005). In light of these observations,

it is possible that at least some of the target genes normally regulated by NOR are also responsive to wounding and thus would have been eliminated in our analysis given the nature of our wounded tissue control. The NOR protein itself cannot be directly responsible for the increase in gene expression observed in *nor* wounded fruit since this protein is not functional in those fruits. It is however possible that other transcription factors, such as other NACs, might mediate wounding induced expression of these genes.

A second explanation for our negative result is the relatively low statistical power of our microarray experiment. Even if a quantitative difference exists between gene expression in induced *pVNF* tissue and *nor* wounded tissue, the limited number of replicates used in the experiment may have impaired our ability to detect any statistically significant differences in gene expression. Consistent with this idea, *NOR*, *RIN*, *ACS4*, *E8* showed about a two-fold increase in expression between the wounded tissue and the *NOR*-induced tissue, but this difference was not significant based on the p-value. Lowering this stringency requirement to $p<0.2$ still failed to yield more candidate target genes.

4.5 CONCLUSION

Although the approach described above failed to generate conclusive results, it provided useful observations that could be used to design an alternative system and/or experiments better suited to study the targets of RIN and NOR during fruit ripening. The use of a different cultivar, such as Microtom, and the expression of an untagged version of the RIN and NOR proteins, might prove to be a more effective strategy.

CHAPTER 5 EXPRESSION OF A GFP-TAGGED TDR4 TRANSGENE IN TOMATO

5.1 INTRODUCTION

Fruit ripening requires the activation and coordination of numerous pathways. As demonstrated in the previous chapters, the transcription factors RIN and NOR play critical roles in the initiation of tomato fruit ripening by regulating the expression of target genes. The MADS box *TDR4* gene is among the transcription factors whose expression seems to be influenced by RIN and NOR activities during ripening and work by others in the lab suggest that RIN and TDR4 are capable of direct interaction in a Y2H assay (J. Vrebalov, pers. comm.). TDR4 belongs to the SQUA lineage of plant MADS box proteins, named after the *A.majus* SQUAMOSA encoding gene. Members of this subfamily are known principally for their role in floral meristem identity establishment (Huijser et al., 1992; Saedler and Huijser, 1993), but have also been shown to be involved in potato tuber formation (Rosin et al., 2003) and *A.thaliana* fruit development (Dinneny et al., 2005). The *FRUITFULL (FUL)* gene has been shown to regulate cell differentiation in the siliques of *A. thaliana*. Loss of function mutations in this gene results in the production of short and compact siliques that fail to dehisce properly (Gu et al., 1998).

A recent study uncovered a role for a TDR4 ortholog in bilberry (*Vaccinium myrtillus*) fruits (Jaakola et al., 2010). Silencing of the *VmTDR4* gene leads to significantly reduced accumulation of anthocyanin in these fruits and reduction in the expression of several genes involved in anthocyanin biosynthesis in the silenced *VmTDR4* berries further suggests that TDR4 acts as regulator of the anthocyanin synthesis. The *A.*

thaliana FUL gene has also been shown to influence anthocyanin production under certain conditions through its regulation of the *MYB* transcription factor *PAP2* (G. Seymour, pers.comm.). Interestingly, over-expression of the tomato *TDR4* gene in *A.thaliana* induces the accumulation of anthocyanin in the siliques (Jaakola et al., 2010). Tomato fruits only produce significant amounts of anthocyanin in the epidermis, therefore the role played by *TDR4* during ripening remains unclear. Experiments reported by Lozano (2009) suggest a non ripening phenotype in tomato *TDR4* silenced lines (Lozano et al., 2009) suggesting that this gene is a key component of ripening, although no data were shown. This report however contradicts observation made by Jaakola et al. (2010) who did not observe any phenotype in antisense*TDR4* lines.

Expression studies in tomato have shown that *TDR4* is expressed in both flowers and fruits. In the flower, *TDR4* expression is detected in the young floral meristem, suggesting a role in floral meristem identity and in the carpel and ovules of flowers at anthesis (Pnueli et al., 1991; Lozano et al., 1998; Busi et al., 2003). During fruit development, the expression of *TDR4* increases shortly before the beginning of the ripening program, peaks around the BK stage, and decreases slowly during the remainder of the ripening period (Eriksson et al., 2004).

In order to better understand the role of this transcription factor during tomato ripening, I generated transgenic tomato lines expressing a tagged version of the *TDR4* gene.

5.2 MATERIAL AND METHODS

Plants

Transgenic plants were generated in the tomato cultivar Ailsa Cragi. All plants were grown under normal greenhouse condition until maturity. Fruits were staged based on the number of days from anthesis to breaker stage as defined by the detection of orange coloring at the base of wt fruits.

BAC screening

A tomato MboI BAC collection (Mueller et al., 2005) was screened using a TDR4 specific probe (Giovannoni et al., 1995).

DNA constructs

Construction of the vector pTTE was made in several steps outlined below:

1) The TDR4 promoter was PCR amplified in three overlapping fragments with the following primers: fragment 1 (TDR4-pr1F : 5'-TATAAACGCTTGCG-GATAACTTTAAGAGATTACAAATGAC-3', TDR4-pr1R: 5'-TACAAGC-GCC-ATAGTATGCTCGGA-3'), fragment 2 (TDR4-pr2F : 5'-CCGAAATA-TTGCCTACCAAACACCC-3', TDR4 pr2R : 5'-AGTAGACAATAGCCG-TGCGCATCT-3'), fragment 3(TDR4pr3F : 5'-GGCTTCGAAACTATGTA-GAGGCCA-3', TDR4pr3 R : 5'- CAAA-AACAACAGGATCTATCGAG-3'). Each of these fragments was cloned individually into pGEM-Teasy vector (Promega). The fragments were then assembled into pGEM-Teasy to form the full promoter (pGEM-TDR4pr) using *KpnI*, *MfeI* and *BglII* enzymes(NEB) to make pGEM-TDR4pr.

2) TDR4 cDNA was PCR amplified (TDR4F: 5'-TATAAAGCTTAAAATGGG-AAGAGG-3', TDR4R : 5'-TATAGAGCTCATTATTAAGATGACG-3'), digested with *HindIII* and *SacI* enzymes (NEB) and cloned into pGEM-Teasy predigested with the same enzymes to make the vector pGEM-TDR4

3) The full-length TDR4 promoter was subcloned into pGEM-TDR4 using *BglII* and *SacII* enzymes to give pGEM-TDR4pr::TDR4

4) TDR4pr::TDR4 was subcloned into pBi101 vector using the restriction enzymes *HindIII* and *SacI* (NEB) to create pBi-TDR4pr::TDR4.

5) EGFP tag was amplified from the FULLpr::FUL-EGFP vector (G. Seymour) using the primers EGFP F (5'-TATAGAGCTCCATGGTGAGCAAGGGCG-AGGAGCT-G-3') and EGFP R (5'-TATAGAATTCTTACTTGTACAGCT-CGTCCATG-3'), digested with the *EcoRI* and *SacI* enzymes and cloned into pBi-TDR4p::TDR4 predigested with the same enzymes to create pTTE.

RT-PCR

RNA extracted from tomato flowers was used for reverse transcription using the SuperScript III kit and following the manufacturer's instructions (Invitrogen). The resulting cDNA was used in a PCR reaction with the TDR4 and EGFP specific primers listed above.

Western Blot Analysis

Proteins were extracted from floral and fruit tissue as described in Chapters 2 and 3, separated on SDS-PAGE gels and transfer to nitrocellulose membrane. Western blot analysis was performed using anti-GFP from Sigma (G1544).

5.3 RESULTS

5.3.1 Promoter cloning

In order to study the role of *TDR4*, I expressed a GFP-tag version of the protein in transgenic tomatoes. Based on previous attempts at generating transgenic *TDR4* lines (G. Seymour, pers. comm.), transgene expression was controlled by the endogenous TDR4 promoter to avoid any deleterious effect that might be caused by ectopic

expression of this gene. At the time this project was initiated, the tomato genome sequence had not yet been released and the 5' upstream sequence of the *TDR4* gene was unknown. A BAC screening approach was therefore used to isolate the *TDR4* promoter. A probe corresponding to the *TDR4* cDNA was used to screen BAC libraries. Three putative BACs were isolated and analysed by PCR to confirm the presence of the *TDR4* gene. Only one clone was confirmed to carry the desired genomic region and was used for sequencing, resulting in a region of 2 kb upstream of the transcription start site (TSS).

5.3.2 Promoter analysis

Examination of the *TDR4* promoter region using available *in silico* analysis tools, such as PlantCare and PLACE, revealed the presence of numerous putative *cis*-elements. I also performed a manual search for other elements more relevant to this study. The locations of the *MADS* box *CArG* motif, the *NACBS*, the newly characterized putative *RIN* binding site, and the *CGTA* motif bound by *SQUAMOSA BINDING PROTEIN (SBP)* is depicted in Figure 5-1A. Surprisingly, no *NACBS* motif was found in this region of the *TDR4* promoter; however several putative *SBP* binding motifs were present, suggesting that a member of the SBP-like family could regulate its expression, a good candidate being the CNR protein (Manning et al., 2006). Putative MADS box and RIN binding motifs were also found in this promoter. Further experimentation will be needed to address the importance of these motifs in *TDR4* regulation.

5.3.3 Generation of transgenic lines

In order to generate tagged TDR4 expression transgenic lines, the 2 kb promoter region of *TDR4*, the *TDR4* cDNA and a C-terminal *EGFP* tag were cloned in the pBi101 vector. This construct was then transformed into tomato (cv Ailsa Craig). A

total of eight transformed lines containing the construct were recovered and the plants grown to maturity. No obvious fruit phenotypes were observed in half of the lines grown. The other half of the transformed plants showed flowers senescing before reaching the anthesis stage (Figure 5-1B). As a result of this phenotype, no fruit and no seeds could be recovered from these plants. In order to determine if this floral phenotype could be the result of *TDR4* expression in the flower, RT-PCR analysis was performed. Whole flowers were collected before they began senescing and their RNA was extracted. Figure 5-1 C shows that the *TDR4-EGFP* transcript is specifically detected in *pTTE* lines, but not in non-transformed lines. A Western blot analysis was next used to verify the presence of the protein, but no protein could be detected in the flowers using an anti-GFP antibody (Figure 5-1D). Similarly, the expression of the TDR4-EGFP protein was assessed in the lines producing fruits, but TDR4-EGFP protein was not detected in BK stage transgenic fruits (Figure 5-1E). For one line, a 30 kDa band was detected in the fruit but this likely corresponds to the EGFP moiety alone (estimate size 30kDa) rather than the TDR4-EGFP fusion, whose predicted size is approximately 60kDa.

5.4 DISCUSSION

In order to study the role of the TDR4 MADS box proteins role in fruit ripening, I generated transgenic tomato lines expressing a GFP-tagged version of TDR4. I used the endogenous *TDR4* promoter to drive the expression of this transgene since a previous attempt suggested that constitutive expression of TDR4 using the 35S promoter was unviable (G. Seymour, pers. comm.). However, none of the transgenic fruits obtained from our activity expressed the TDR4-GFP protein. There are several possible explanations for this negative result.

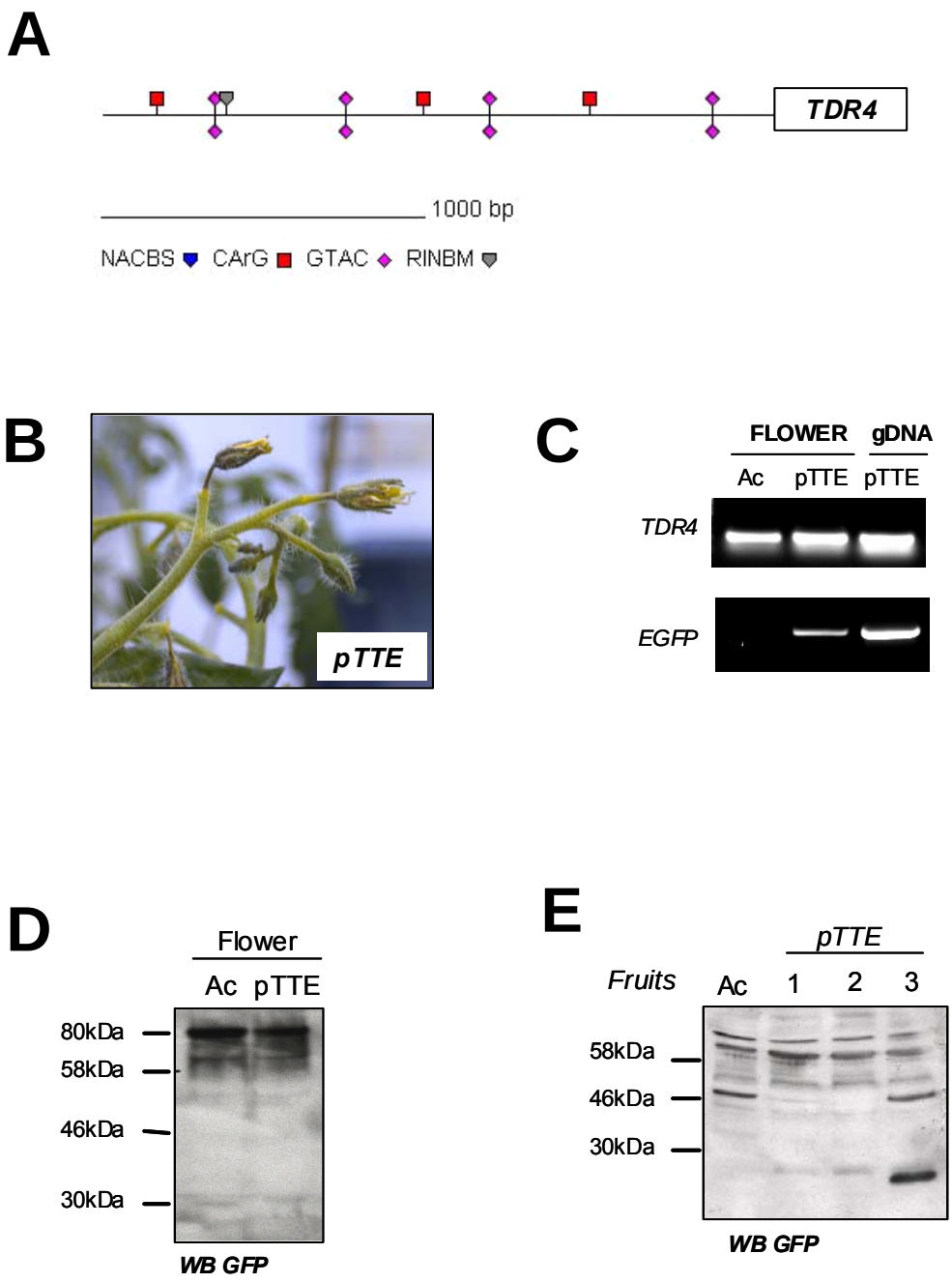


Figure 5-1 TDR4 transgenic lines A) schematic representation of a TDR4 2kb promoter with the location of relevant cis-elements. B) Floral phenotype seen in half of pTTE transgenic lines. C) TDR4 and EGFP-specific RT-PCR of Ac and pTTE flowers. D)E) GFP Western blot analysis of protein isolated from Ac and pTTE flowers (D) and fruits (E)

5.4.1 Floral phenotype

About 50% of the transgenic lines obtained did not produce fruits due to early senescence of their flowers (Fig 5.3-1B). Since the *TDR4* gene is known to be expressed in developing flowers (Pnueli et al., 1991; Lozano et al., 1998; Busi et al., 2003), it is possible that the expression of the transgene in the floral tissue is responsible for the observed floral phenotype. A RT-PCR experiment demonstrated that the transgene is indeed expressed in the flowers before they senesce, but no protein could be detected using a GFP Western blot analysis. This phenotype might have led to the loss of the transgenic lines expressing higher level of *TDR4* transgene.

5.4.2 Promoter sequence

The absence of TDR4 protein in the fruit of the remaining lines analysed could be due to the promoter selected to drive expression. The 2kb *TDR4* promoter region used might not contain all of the *cis* elements required for proper expression of the gene in tomato fruits. A 2kb promoter is often sufficient for proper spatio-temporal expression, for example, the expression of the E8 gene in fruit at the onset of ripening is regulated by 2.1kb promoter (Deikman and Fischer, 1988; Deikman et al., 1992; Deikman et al., 1998). However, a number of examples however indicate that this is not always the case. The functional promoter region of the *PG2a* gene was shown to extend up to 4.5kb upstream of the TSS (Nicholass et al., 1995). It is possible that the promoter required for the proper expression of *TDR4* in fruit requires more than the 2kb used here. In addition to the upstream sequences, regions downstream of the TSS, such as introns and 3' regions, can influence gene transcription (Gidekel et al., 1996; de Boer et al., 1999; Dorsett, 1999). Of particular relevance for this study, previous work reveals that the introns of MADS box genes often play a critical role in controlling their correct spatial and temporal expression. The expression of the

MADS box gene *AGAMOUS* (*AG*) in *A. thaliana* is regulated in part by a *cis*-element located in its second intron of the gene (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth, 2000), and the the first intron of *SEP3* was shown to be essential for correct gene expression (de Folter et al., 2007). Further analysis of the 5' upstream and intronic regions of the *TDR4* gene might reveal other *cis*-elements that are important for fruit expression. Ideally, characterization of the *TDR4* promoter should involve the GUS reporter system to avoid any deleterious effect associated with the ectopic expression of the *TDR4* gene.

CHAPTER 6 SUMMARY AND FUTURE DIRECTIONS

6.1 RIN AND NOR INTERACTION NETWORKS

The work presented in this dissertation shed light on the role of the *RIN* and *NOR* genes in regulating ripening. The molecular interactions described in Chapters 2 and 3 provide a general picture of the transcriptional network involved in ripening. The model presented in Figure 6-1 summarizes these events. After reaching the mature green stage, an unknown activity activates the transcription of the NOR gene which leads to the transcription of the *RIN* gene possibly through an indirect mechanism - since no *in vivo* interaction could be detected between the *RIN* promoter and the NOR protein using the ChIP assay (Chapter 3). The NOR protein is also recruited to the promoters of other target genes, including *NR*, *PG2a* and *E8*. The RIN protein likely associates with CNR-regulated protein(s) to form a DNA binding-competent complex that is then recruited to numerous target genes, including ethylene synthesis and signaling genes (*ACS2, ACS4, NR, E8, E4*), transcription factor genes (*NOR, RIN, HB1, CNR)*, cell wall modifying genes (*PG2a, Ext*) and at least one rate limiting carotenoids biosynthetic gene (*PSY1*). Expression of these genes and others lead to the physiologic changes associated with ripening.

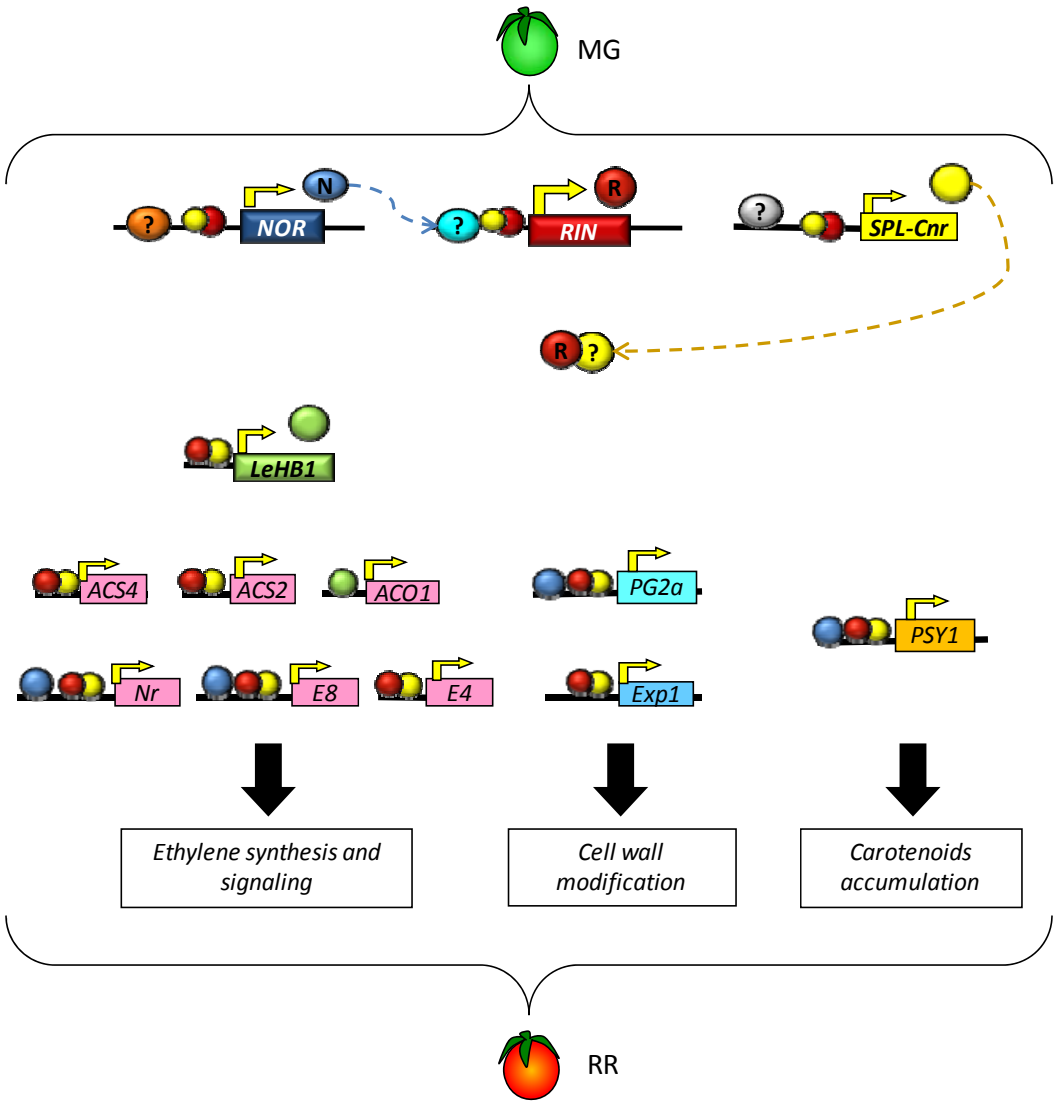


Figure 6-1 Role of RIN and NOR in tomato fruit ripening. Putative interaction network involved in fruit ripening. An unknown factor ("?" orange circle) activates the expression of *NOR* at the onset of ripening. A NOR-dependant protein ("?" light blue circle) activates the expression of *RIN*. An unknown factor ("?" gray circle) activates the expression of the *CNR* gene. RIN protein (red circle) interacts with a CNR-dependant protein ("?" yellow circle). *HB1* protein is depicted as green circle. Primary target genes are depicted as colored rectangles. Association of the NOR protein to primary target is illustrated by blue circle, association of a RIN complex to a primary target is illustrated by red and yellow circles. Dash lines indicate indirect connection. MG: Mature green fruit, RR : red ripe fruit.

6.2 RIN AND NOR DNA ASSOCIATION MODELS

The model presented above depicts the interaction of RIN and NOR with the genes they directly regulate, however the exact mechanism by which both protein bind to their primary targets remains speculative. Two mechanistic models can be proposed to explain the recruitment the transcription factor to its target promoter.

In model 1, the transcription factor is recruited by directly binding DNA of the target gene promoter. RIN and NOR both belong to well characterized transcription factor families whose target *cis*-elements are known. MADS box proteins can directly interact with CArG box motifs (Messenguy and Dubois, 2003). However, the absence of RIN binding to the CArG box *in vitro* and the lack of correlation between the presence of this motif and our ChIP data suggest that this model is unlikely to account for much of the RIN interaction detected. An alternative possibility is that RIN binds directly to DNA throught a *cis*-elements other than a CArG motif. A search for other over-represented motifs present in the promoter of the RIN-ChIPed genes led to the identification of a novel motif whose presence correlates tightly with RIN association. It is possible that RIN recruitment to some target promoters is mediated by direct binding to this motif, however direct interaction of other MADS box proteins with non-CArG box element has not been reported and, therefore, make this model tantalizing based on available data yet speculative pending further verification.
As a member of the NAC domain transcription factor family, NOR interaction with DNA might be expected to be mediated by direct binding to the NACBS motif (Olsen et al., 2005). In Chapter 3, I indeed demonstrated the ability of NOR to bind the NACBS motif *in vitro* and further showed strong correlation between *in vitro* NACBS binding and *in vivo* NOR association. Taken together these results strongly support a

model in which NOR interaction with its target genes is mediated by direct binding to a NACBS motif. Whether this direct binding model is responsible for all NOR-target genes association remains to be demonstrated. It is possible that NOR recruitment to promoters is mediated by more than one mechanism.

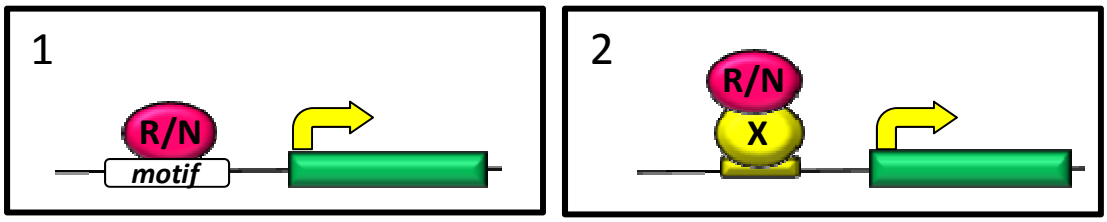


Figure 6-2 Mechanistic promoter recruitment models. In model 1, RIN or NOR proteins (R/N, pink circles) associate directly with their target promoter by binding to a specific *cis*-element ("motif" white box). In model 2, RIN or NOR are recruited to their target promoters through protein-protein interaction with one or more other factors ("X" yellow cirlce) that presumably interact with specif *cis*-elements (yellow box) motif(s). Green box represent target gene.

In model 2, RIN and NOR are recruited to their target gene promoters by associating with other DNA bound factors. Several arguments support this model for RIN-promoter interactions. First, as mentioned above, no correlation between RIN *in vivo* associations and the presence of a CArG box was found, suggesting that RIN does not interact directly with DNA through this motif. Second, I have shown in Chapter 2 that RIN *in vivo* promoter association depends on the presence of a CNR-dependant factor, suggesting that RIN alone is unable to bind to DNA. MADS box proteins are know to form higher molecular weight complexes (Messenguy and Dubois, 2003). The *SEP* proteins, members of the same clade as RIN, have been shown to act as scaffolding proteins enabling the formation of multimeric protein complexes involved in gene regulation during floral organ identity formation (Immink et al., 2009). RIN might be involved in a similar mechanism in ripening, promoting the formation of larger

complexes able to regulate the expression of a large subset of genes. Thus the recruitment of RIN to its target promoter might not depend on its DNA binding activity rather on its ability to interact with other proteins, themselves interacting directly with DNA. The identification of an enriched motif in the promoter sequences of RIN bound targets could help identify such co-factors. As observed in Chapter 2, RIN associates with both ethylene-dependant and ethylene-independent genes. This observation raises the possibility that RIN is recruited to different classes of genes by interacting with different protein complexes. A similar mechanism was suggested to account for SEP3 target interactions (Kaufmann et al., 2009). If more than one RIN-protein complexe exists, it is likely that more than one *cis*-element is responsible for their recruitment.

Although results presented in Chapter 3 strongly support a direct interaction model for NOR, it remain to be confirmed if this mechanism is responsible for all NOR-promoter interactions. It is possible that more than one mechanism is responsible for NOR recruitment to target genes. While I have made very significant progress in defining specific gene targets of RIN and NOR, future studies will be needed to define specific RIN and NOR regulatory interactions.

6.3 FUTURE STUDIES

Several levels of information must be examined to fully understand the role of transcription factors such as RIN and NOR and these can be broadly divided into three, somewhat overlapping, categories: (1) downstream events, that is the identity of the genes and pathways regulated by the transcription factors (TF); (2) *in trans* events, relating to protein functions including other interacting proteins, DNA-binding activities and post-translational modifications; and (3) upstream events, i.e. regulatory

mechanisms involved in the transcriptional and post-transcriptional (e.g. miRNA) regulation of the expression of the TFs themselves. I focused principally on the first category to understand the role of RIN and NOR in ripening in the context of direct promoter interactions and corresponding gene targets. Several additional experiments could help clarify and expand our knowledge of RIN and NOR targets. However, experiments addressing the other two categories of events are equally important to fully understand the function of these proteins during fruit ripening. Following are some suggested experiments that could be undertaken to further explore the developmental/TF-mediated regulation of fruit ripening.

6.4 DOWNSTREAM EVENTS

6.4.1 ChIP-Seq with RIN and NOR antibodies

A main goal of this dissertation was the identification of *RIN* and *NOR* primary promoter targets. Several approaches were undertaken to address this phenomena including microarray analyses, chromatin immunoprecipitations, *in vitro* electromobility shift assays and the development of an inducible transgenic tomato system. These approaches revealed some of the primary targets of RIN and NOR. However, a more thorough identification of these targets is required to obtain a more complete picture of the transcriptional network regulated by these key transcription factors. The recent development and increasing affordability of next generation sequencing techniques such as Solexa/Illumina and 454 (Hawkins et al., 2010), now allow more comprehensive transcriptome profiling. By coupling the chromatin immunoprecipiation technique developed in the current dissertation with these high-throughput sequencing approaches (so-called CHiP-seq), one should be able to generate a more detailed picture of the targets directly regulated by RIN and NOR

(Kaufmann et al., 2006). Such an approach has been successfully used to identify the targets of other MADS box genes including *SEP3* and *AP1* in *A. thaliana* (Kaufmann et al., 2003; Kaufmann et al., 2009).

6.4.2 Inducible system

Although I failed to obtain significant results from the inducible systems, this approach still has the potential to answer many questions regarding the activity of RIN and NOR. A second generation inducible system should be developed, keeping in mind the negative results described here as guidelines. A first major change that would allow a more effective use of this system would be to introgress the *rin* and *nor* mutations into a more convenient cultivar background, such as Microtom. This cultivar has been used with success for transient expression, virus induced gene silencing and inducible assays (Estornell et al., 2009; Fernandez et al., 2009; Orzaez and Granell, 2009; Orzaez et al., 2009), and Microtom lines of *rin* and *nor* would be valuable tools for numerous assays. Another modification that would enable a greater flexibility of the inducible system would be to use a 1-component instead of a 2-component system. In a 1-component system the protein domain responsible for the inducer responsiveness is added directly to the transcription factor of interest. This enables a quicker response following addition of the inducer. More importantly, this system allows for the simultaneous use of translation inhibiting drugs, such as cycloheximide. By blocking protein synthesis, one can be assured that transcriptomic changes resulting from induction are due exclusively to the transcription factor studied, therefore allowing a more efficient identification of primary targets (Zhang, 2003). One caveat of this approach is that it involves the expression of chimeras of RIN and NOR proteins and the results presented in Chapter 4 suggest that RIN and NOR activity is affected by the addition of tags. As such, it would be important to

verify the functionality of RIN and NOR chimeras before undertaking the development of a 1-component inducible expression system. One fast way to check the DNA binding ability of a NOR chimera would be to use the *in vitro* gel shift assay developed for NOR. If the addition of a tag does impair proper RIN and NOR function alternate tag variants could be created. If this proves to be a general problem with these proteins it might be preferable to use the 2-component system to express a non-tagged version of the gene.

6.5 *IN TRANS* EVENTS

6.5.1 RIN and NOR protein complexes

Another interesting theme to pursue in order to get a better understanding of RIN and NOR functions is the identity of their protein co-factors. It is well known that transcription factors often act as part of multi-protein complexes. This fact has been particularly well established for MADS box proteins (Theissen, 2001). A few studies also characterized the interactions of NAC proteins with other types of factors including MADS box proteins (Olsen et al., 2005). It would be valuable to identify the interacting partners of RIN and NOR and several approaches could be used to address this question. First, a yeast-two-hybrid screen using a tomato fruit specific cDNA library could enable the identification of several of these partners. Since some protein-protein interactions involve more than one partner (Immink et al., 2009), an *in vivo* approach could be used to capture others. Isolation of *in vivo* protein complexes containing RIN and NOR could be attempted using immunoprecipitation with the antibodies developed in this dissertation. Alternatively, a tap-tag purification approach (Van Leene et al., 2008) could be employed.

A candidate gene approach could also be considered in light of the results obtained in this study. I showed that RIN transcriptional activity is defective in the *cnr* mutant even if the protein is still present. One question raised by this observation is whether RIN and CNR are part of the same complex. Regarding the NOR protein, one important characteristic of NAC proteins is their need for dimerization. Consequently, it would be interesting to test whether the NOR protein might be able to heterodimerize with additional fruit expressed NAM-like proteins (see Chapter 1). Both RIN and NOR candidate interactions could be tested by a series of approaches including GST-pulldown assays, yeast-two-hybrid analysis and immunoprecipitation.

6.5.2 Post-translational regulation

An interesting observation from this study is the difficulty in detecting NOR protein in non-fruit tissues. I failed to detect NOR protein in induced transgenic *pVNF* leaves although the mRNA could clearly be assayed which suggests that the NOR protein might be unstable, or not translated, when expressed outside the fruit. Prior studies report ubiquitin-mediated degradation of NAC proteins during normal development (Xie et al., 2002) and as a mechanism of viral suppression of host defense (Wang et al., 2009; Yoshii et al., 2010). It would be interesting to determine if the expression of NOR is controlled by protein degradation. Experiments using the proteasome inhibitor MG132 and Western blot analysis using antibodies recognizing ubiquitin could help reveal any role of this mechanism in NOR protein stability.

6.5.3 Characterization of NOR preferred binding site

The ability of NOR to bind to the core NACBS motif *in vitro* is described in Chapter 3. Interestingly not all the putative NACBS tested were bound by NOR, which indicates a difference in motifs affinities. As mentioned before, NAC proteins show specific binding preferences for the nucleotides surrounding the core “CCGT” motif

(Olsen et al., 2005). Techniques such as CASTing (Cyclic Amplification and Selection of Targets), which involves several cycles of *in vitro* binding of the purified protein of interest (here NOR) to a pool of degenerated double stranded oligonucleotides (Pierrou et al., 1995), have been used to characterize transcription factor binding motifs of certain NAC proteins (Olsen et al., 2005; Yabuta et al., 2010). A similar characterization for NOR would allow for a more efficient identification of candidate target genes.

6.5.4 *In planta* transcriptional assay

In addition to *in vitro* assays, it would be interesting to confirm the interactions of NOR with its target *cis*-elements *in vivo*. A few papers have reported the successful use of transient expression assays in tomato fruit (Orzaez et al., 2006; Orzaez and Granell, 2009). Although previous attempts to replicate this work in our lab have largely proven unsuccessful, the optimization of this technique would definitively provide a useful tool for the rapid characterization of promoter sequences involved in expression of specific genes during ripening. Another *in planta* approach that would provide useful information concerning the *in vivo* DNA binding ability of RIN and NOR is the dual luciferase assay (Hellens et al., 2005). In this assay, *N. benthamiana* is used for co-transient expression of a transcription factor of interest and a reporter construct composed of a specific promoter region driving the expression of a luciferase reporter gene. This system was successfully used to indentify MYB genes involved in the regulation of anthocyanin in apples (Espley et al., 2007) and the optimization of this system would enable us to test the binding ability of RIN and NOR to candidate promoters.

6.6 UPSTREAM EVENTS

6.6.1 Post-transcriptional regulation

Several *NAC* and *MADS* box genes have been shown to be regulated at the post transcriptional level by miRNAs. Expression of *Z. mays* and *A. thaliana AP2* is negatively regulated by miRNA 172 during floral development (Zhao et al., 2007; Zhu et al., 2009); and similar regulation has been observed for *A. thaliana AGL16* by miRNA824 during stomatal development (Kutter et al., 2007). *NAC* genes that are regulated by miRNA includes the tomato *GOB* gene (Berger et al., 2009) , and the *A. thaliana NAC1* (Guo et al., 2005) and *CUC2* (Mallory et al., 2004; Baker et al., 2005; Raman et al., 2008; Larue et al., 2009) genes. Interestingly, all of the miRNA-regulated NACs characterized to date involve miRNA164. An examination of the *NOR* sequence indicates that it does not possess a recognition region for this specific miRNA. This suggests that *NOR* expression is either not regulated by miRNAs or that a miRNA, other than miRNA164, is involved in its regulation. Tomato miRNAs expressed during fruit ripening were recently identified (Moxon et al., 2008) which should help define the role, if any, that miRNAs play in the regulation of RIN and/or NOR activities. A potential first step would be to identify potential miRNA recognition sites in *RIN* and *NOR* sequences. If such sites were found, generating transgenic plants expressing miRNA –insensitive versions of these genes would allow researchers to study the implications of this posttranslational regulatory mechanism during ripening

6.6.2 RIN and NOR promoter analysis

The experiments described above should lead to a better understanding of the function on RIN and NOR proteins at the onset of ripening. However, the question of what is

responsible for the temporal expression of these genes at the end of the fruit developmental phase remains. In order to answer this question, it may be helpful to study the *RIN* and *NOR* promoters. A number of promoter::GUS transgenic plants are currently being analyzed with the goal of defining the genomic regions sufficient for the proper expression of these genes. Once those regions are more precisely defined, a fine mapping of the *cis*-elements important for transactivation at the onset of ripening should be undertaken. The optimization of the fruit transient expression assay described in Chapter 4 would greatly increase the efficiency of such a characterization. Another approach that could be considered to identify important promoter regions of the *RIN* and *NOR* promoters is phylogenetic shadowing (Boffelli et al., 2003), Adrian et al., *in press*). This technique involves the comparison of the sequences of orthologous promoters between closely related species. Regions of greater similarity are likely to contain *cis*-elements important for gene expression. This approach has been used to identify *cis*-elements in the promoter of the *A. thaliana* MADS box gene *AG* (Hong et al., 2003). For the purpose of identifying *RIN* and *NOR cis*-elements, a comparison between their promoters in *S. lycopersicum* and its wild relatives (e.g. *S. pennellii, S. pimpinellifolium, S. galapagense*) could be considered. It would however be important to first verify that *RIN* and *NOR* genes are expressed during ripening in the fruits of these species.

REFERENCES

Aarts MG, Keijzer CJ, Stiekema WJ, Pereira A (1995) Molecular characterization of the CER1 gene of arabidopsis involved in epicuticular wax biosynthesis and pollen fertility. Plant Cell **7:** 2115-2127

Adams-Phillips L, Barry C, Giovannoni J (2004) Signal transduction systems regulating fruit ripening. Trends Plant Sci **9:** 331-338

Adams-Phillips L, Barry C, Kannan P, Leclercq J, Bouzayen M, Giovannoni J (2004) Evidence that CTR1-mediated ethylene signal transduction in tomato is encoded by a multigene family whose members display distinct regulatory features. Plant Mol Biol **54:** 387-404

Adams DO, Yang SF (1977) Methionine Metabolism in Apple Tissue: Implication of S-Adenosylmethionine as an Intermediate in the Conversion of Methionine to Ethylene. Plant Physiol **60:** 892-896

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997) Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. Plant Cell **9:** 841-857

Alba R, Fei Z, Payton P, Liu Y, Moore SL, Debbie P, Cohn J, D'Ascenzo M, Gordon JS, Rose JK, Martin G, Tanksley SD, Bouzayen M, Jahn MM, Giovannoni J (2004) ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development. Plant J **39:** 697-714

Alba R, Payton P, Fei Z, McQuinn R, Debbie P, Martin GB, Tanksley SD, Giovannoni JJ (2005) Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. Plant Cell **17:** 2954-2965

Alexander L, Grierson D (2002) Ethylene biosynthesis and action in tomato: a model for climacteric fruit ripening. J Exp Bot **53:** 2039-2055

Alonso JM, Hirayama T, Roman G, Nourizadeh S, Ecker JR (1999) EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science **284:** 2148-2152

Alvarez-Buylla ER, Liljegren SJ, Pelaz S, Gold SE, Burgeff C, Ditta GS, Vergara-Silva F, Yanofsky MF (2000) MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. Plant J **24:** 457-466

Alvarez-Buylla ER, Pelaz S, Liljegren SJ, Gold SE, Burgeff C, Ditta GS, Ribas de Pouplana L, Martinez-Castilla L, Yanofsky MF (2000) An ancestral MADS-box gene duplication occurred before the divergence of plants and animals. Proc Natl Acad Sci U S A **97:** 5328-5333

Angenent GC, Franken J, Busscher M, Weiss D, van Tunen AJ (1994) Co-suppression of the petunia homeotic gene fbp2 affects the identity of the generative meristem. Plant J **5:** 33-44

Bai Y, Lindhout P (2007) Domestication and Breeding of Tomatoes: What have We Gained and What Can We Gain in the Future? Ann Bot (Lond)

Bailey TL, Elkan C (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *In* Second International Conference on Intelligent Systems for Molecular Biology. AAAI Press, Menlo Park, California, pp 28-36

Baker CC, Sieber P, Wellmer F, Meyerowitz EM (2005) The early extra petals1 mutant uncovers a role for microRNA miR164c in regulating petal number in Arabidopsis. Curr Biol **15:** 303-315

Balazadeh S, Siddiqui H, Allu AD, Matallana-Ramirez LP, Caldana C, Mehrnia M, Zanor MI, Kohler B, Mueller-Roeber B (2010) A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. Plant Journal **62:** 250-264

Balbi V, Lomax TL (2003) Regulation of early tomato fruit development by the diageotropica gene. Plant Physiology **131:** 186-197

Barry CGJ (2007) Ethylene and fruit ripening. Journal of Plant Growth Regulation **26:** 143-159

Barry CS, Llop-Tous MI, Grierson D (2000) The regulation of 1-aminocyclopropane-1-carboxylic acid synthase gene expression during the transition from system-1 to system-2 ethylene synthesis in tomato. Plant Physiol **123:** 979-986

Bartley GE, Viitanen PV, Bacot KO, Scolnik PA (1992) A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway. J Biol Chem **267:** 5036-5039

Becker A, Theissen G (2003) The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Molecular Phylogenetics and Evolution **29:** 464-489

Becker A, Theissen G (2003) The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol **29:** 464-489

Berger Y, Harpaz-Saad S, Brand A, Melnik H, Sirding N, Alvarez JP, Zinder M, Samach A, Eshed Y, Ori N (2009) The NAC-domain transcription factor GOBLET specifies leaflet boundaries in compound tomato leaves. Development **136:** 823-832

Bleecker AB, Kende H (2000) Ethylene: a gaseous signal molecule in plants. Annu Rev Cell Dev Biol **16:** 1-18

Blume B, Grierson D (1997) Expression of ACC oxidase promoter-GUS fusions in tomato and Nicotiana plumbaginifolia regulated by developmental and environmental stimuli. Plant J **12:** 731-746

Boffelli D, McAuliffe J, Ovcharenko D, Lewis KD, Ovcharenko I, Pachter L, Rubin EM (2003) Phylogenetic shadowing of primate sequences to find functional regions of the human genome. Science **299:** 1391-1394

Bohner J, Bangerth F (1988) Effects of Fruit-Set Sequence and Defoliation on Cell Number, Cell-Size and Hormone Levels of Tomato Fruits (Lycopersicon-Esculentum Mill) within a Truss. Plant Growth Regulation **7:** 141-155

Bowman JL, Meyerowitz EM (1991) Genetic control of pattern formation during flower development in Arabidopsis. Symp Soc Exp Biol **45:** 89-115

Busi MV, Bustamante C, D'Angelo C, Hidalgo-Cuevas M, Boggio SB, Valle EM, Zabaleta E (2003) MADS-box genes expressed during tomato seed and fruit development. Plant Mol Biol **52:** 801-815

Caddick MX, Greenland AJ, Jepson I, Krause KP, Qu N, Riddell KV, Salter MG, Schuch W, Sonnewald U, Tomsett AB (1998) An ethanol inducible gene switch for plants used to manipulate carbon metabolism. Nat Biotechnol **16:** 177-180

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene-response gene ETR1: similarity of product to two-component regulators. Science **262:** 539-544

Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89:** 1133-1144

Chen G, Hackett R, Walker D, Taylor A, Lin Z, Grierson D (2004) Identification of a specific isoform of tomato lipoxygenase (TomloxC) involved in the generation of fatty acid-derived flavor compounds. Plant Physiol **136:** 2641-2651

Chen X (2004) A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development. Science **303:** 2022-2025

Chen X, J.L. R, Jia D, E. M (2000) Minimal regions in the Arabidopsis PISTILLATA promoter responsive to the APETALA3/PISTILLATA feedback control do not contain a CArG box. Sexual Plant Reproduction **13:** 85-94

Cheniclet C, Rong WY, Causse M, Frangne N, Bolling L, Carde JP, Renaudin JP (2005) Cell expansion and endoreduplication show a large genetic variability in pericarp and contribute strongly to tomato fruit growth. Plant Physiology **139:** 1984-1994

Cho SC, Jang SH, Chae SJ, Chung KM, Moon YH, An GH, Jang SK (1999) Analysis of the C-terminal region of Arabidopsis thaliana APETALA1 as a transcription activation domain. Plant Molecular Biology **40:** 419-429

Clark KL, Larsen PB, Wang X, Chang C (1998) Association of the Arabidopsis CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. Proc Natl Acad Sci U S A **95:** 5401-5406

Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. Nature **353:** 31-37

Collinge M, Boller T (2001) Differential induction of two potato genes, Stprx2 and StNAC, in response to infection by Phytophthora infestans and to wounding. Plant Mol Biol **46:** 521-529

Cordes S, Deikman J, Margossian LJ, Fischer RL (1989) Interaction of a developmentally regulated DNA-binding factor with sites flanking two different fruit-ripening genes from tomato. Plant Cell **1:** 1025-1034

Craft J, Samalova M, Baroux C, Townley H, Martinez A, Jepson I, Tsiantis M, Moore I (2005) New pOp/LhG4 vectors for stringent glucocorticoid-dependent transgene expression in Arabidopsis. Plant J **41:** 899-918

Cunningham FX, Gantt E (1998) Genes and Enzymes of Carotenoid Biosynthesis in Plants. Annu Rev Plant Physiol Plant Mol Biol **49:** 557-583

Davies B, Motte P, Keck E, Saedler H, Sommer H, Schwarz-Sommer Z (1999) PLENA and FARINELLI: redundancy and regulatory interactions between two Antirrhinum MADS-box factors controlling flower development. EMBO J **18:** 4023-4034

de Boer GJ, Testerink C, Pielage G, Nijkamp HJ, Stuitje AR (1999) Sequences surrounding the transcription initiation site of the Arabidopsis enoyl-acyl carrier protein reductase gene control seed expression in transgenic tobacco. Plant Mol Biol **39:** 1197-1207

de Folter S, Urbanus SL, van Zuijlen LG, Kaufmann K, Angenent GC (2007) Tagging of MADS domain proteins for chromatin immunoprecipitation. BMC Plant Biol **7:** 47

Deikman J, Fischer RL (1988) Interaction of a DNA binding factor with the 5'-flanking region of an ethylene-responsive fruit ripening gene from tomato. EMBO J **7:** 3315-3320

Deikman J, Kline R, Fischer RL (1992) Organization of Ripening and Ethylene Regulatory Regions in a Fruit-Specific Promoter from Tomato (Lycopersicon esculentum). Plant Physiol **100:** 2013-2017

Deikman J, Xu R, Kneissl ML, Ciardi JA, Kim KN, Pelah D (1998) Separation of cis elements responsive to ethylene, fruit development, and ripening in the 5'-flanking region of the ripening-related E8 gene. Plant Mol Biol **37:** 1001-1011

Dellapenna D, Lincoln JE, Fischer RL, Bennett AB (1989) Transcriptional Analysis of Polygalacturonase and Other Ripening Associated Genes in Rutgers, rin, nor, and Nr Tomato Fruit. Plant Physiol **90:** 1372-1377

Deyholos MK, Sieburth LE (2000) Separable whorl-specific expression and negative regulation by enhancer elements within the AGAMOUS second intron. Plant Cell **12:** 1799-1810

Dinneny JR, Weigel D, Yanofsky MF (2005) A genetic framework for fruit patterning in Arabidopsis thaliana. Development **132:** 4687-4696

Ditta G, Pinyopich A, Robles P, Pelaz S, Yanofsky MF (2004) The SEP4 gene of Arabidopsis thaliana functions in floral organ and meristem identity. Current Biology **14:** 1935-1940

Dorsett D (1999) Distant liaisons: long-range enhancer-promoter interactions in Drosophila. Curr Opin Genet Dev **9:** 505-514

Dupille E, Sisler, E. (1995) Effect of an ethylene receptor antagonist on plant material. *In* A Ait-Oubahou, El-Otmani, M., ed, Postharvest Physiology, Pathology and Technologies for Horticultural Commodities : Recent Advances. Institut Agronomique & Veterinaire Hassan II, Agadir, pp 294-301

Duval M, Hsieh TF, Kim SY, Thomas TL (2002) Molecular characterization of AtNAM: a member of the Arabidopsis NAC domain superfamily. Plant Mol Biol **50:** 237-248

Egea-Cortines M, Saedler H, Sommer H (1999) Ternary complex formation between the MADS-box proteins SQUAMOSA, DEFICIENS and GLOBOSA is involved in the control of floral architecture in Antirrhinum majus. Embo Journal **18:** 5370-5379

Ehret DL, Ho LC (1986) Effects of Osmotic Potential in Nutrient Solution on Diurnal Growth of Tomato Fruit. Journal of Experimental Botany **37:** 1294-1302

Ehret DL, Ho LC (1986) The Effects of Salinity on Dry-Matter Partitioning and Fruit-Growth in Tomatoes Grown in Nutrient Film Culture. Journal of Horticultural Science **61:** 361-367

Ehret DL, Ho LC (1986) Translocation of Calcium in Relation to Tomato Fruit-Growth. Annals of Botany **58:** 679-688

Eriksson EM, Bovy A, Manning K, Harrison L, Andrews J, De Silva J, Tucker GA, Seymour GB (2004) Effect of the Colorless non-ripening mutation on cell wall biochemistry and gene expression during tomato fruit development and ripening. Plant Physiol **136:** 4184-4197

Ernst HA, Olsen AN, Larsen S, Lo Leggio L (2004) Structure of the conserved domain of ANAC, a member of the NAC family of transcription factors. EMBO Rep **5:** 297-303

Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J **49:** 414-427

Estornell LH, Orzaez D, Lopez-Pena L, Pineda B, Anton MT, Moreno V, Granell A (2009) A multisite gateway-based toolkit for targeted gene expression and hairpin RNA silencing in tomato fruits. Plant Biotechnol J **7:** 298-309

Fan HY, Hu Y, Tudor M, Ma H (1997) Specific interactions between the K domains of AG and AGLs, members of the MADS domain family of DNA binding proteins. Plant J **12:** 999-1010

Fei Z, Tang X, Alba R, Giovannoni J (2006) Tomato Expression Database (TED): a suite of data presentation and analysis tools. Nucleic Acids Res **34:** D766-770

Fei Z, Tang X, Alba RM, White JA, Ronning CM, Martin GB, Tanksley SD, Giovannoni JJ (2004) Comprehensive EST analysis of tomato and comparative genomics of fruit ripening. Plant J **40:** 47-59

Fernandez AI, Viron N, Alhagdow M, Karimi M, Jones M, Amsellem Z, Sicard A, Czerednik A, Angenent G, Grierson D, May S, Seymour G, Eshed Y, Lemaire-Chamley M, Rothan C, Hilson P (2009) Flexible tools for gene expression and silencing in tomato. Plant Physiol **151:** 1729-1740

Fraser PD, Bramley P, Seymour GB (2001) Effect of the Cnr mutation on carotenoid formation during tomato fruit ripening. Phytochemistry **58:** 75-79

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LS, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J **39:** 863-876

Furutani M, Vernoux T, Traas J, Kato T, Tasaka M, Aida M (2004) PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in Arabidopsis embryogenesis. Development **131:** 5021-5030

Gallie DR (2010) Regulated ethylene insensitivity through the inducible expression of the Arabidopsis etr1-1 mutant ethylene receptor in tomato. Plant Physiol **152:** 1928-1939

Gao Z, Chen YF, Randlett MD, Zhao XC, Findell JL, Kieber JJ, Schaller GE (2003) Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of Arabidopsis through participation in ethylene receptor signaling complexes. J Biol Chem **278:** 34725-34732

Garoosi GA, Salter MG, Caddick MX, Tomsett AB (2005) Characterization of the ethanol-inducible alc gene expression system in tomato. J Exp Bot **56:** 1635-1642

Gidekel M, Jimenez B, Herrera-Estrella L (1996) The first intron of the Arabidopsis thaliana gene coding for elongation factor 1 beta contains an enhancer-like element. Gene **170:** 201-206

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: A Developmental Perspective. Plant Cell **5:** 1439-1451

Gillaspy G, Bendavid H, Gruissem W (1993) Fruits - a Developmental Perspective. Plant Cell **5:** 1439-1451

Giovannoni J (2001) Molecular Biology of Fruit Maturation and Ripening. Annu Rev Plant Physiol Plant Mol Biol **52:** 725-749

Giovannoni JJ (2004) Genetic regulation of fruit development and ripening. Plant Cell **16 Suppl:** S170-180

Giovannoni JJ (2007) Fruit ripening mutants yield insights into ripening control. Curr Opin Plant Biol **10:** 283-289

Giovannoni JJ, Noensie EN, Ruezinsky DM, Lu X, Tracy SL, Ganal MW, Martin GB, Pillen K, Alpert K, Tanksley SD (1995) Molecular genetic analysis of the ripening-inhibitor and non-ripening loci of tomato: a first step in genetic map-based cloning of fruit ripening genes. Mol Gen Genet **248:** 195-206

Greve K, La Cour T, Jensen MK, Poulsen FM, Skriver K (2003) Interactions between plant RING-H2 and plant-specific NAC (NAM/ATAF1/2/CUC2) proteins: RING-H2 molecular specificity and cellular localization. Biochem J **371:** 97-108

Gu Q, Ferrandiz C, Yanofsky MF, Martienssen R (1998) The FRUITFULL MADS-box gene mediates cell differentiation during Arabidopsis fruit development. Development **125:** 1509-1517

Guichard S, Gary C, Leonardi C, Bertin N (2005) Analysis of growth and water relations of tomato fruits in relation to air vapor pressure deficit and plant fruit load. Journal of Plant Growth Regulation **24:** 201-213

Guo HS, Xie Q, Fei JF, Chua NH (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. Plant Cell **17:** 1376-1386

Gupta S, Stamatoyannopolous J, Bailey TL, Noble WS (2007) Quantifying similarity between motifs. Genome Biology **8:** R24

Gustafson FG (1936) Inducement of fruit development by growth-promoting chemicals. Proceedings of the National Academy of Sciences of the United States of America **22:** 628-636

Gustafson FG (1939) Auxin distribution in fruits and its significance in fruit development. American Journal of Botany **26:** 189-194

Gustafson FG (1960) Influence of Gibberellic Acid on Setting and Development of Fruits in Tomato. Plant Physiol **35:** 521-523

Hansen JD, Pyee J, Xia Y, Wen TJ, Robertson DS, Kolattukudy PE, Nikolau BJ, Schnable PS (1997) The glossy1 locus of maize and an epidermis-specific cDNA from Kleinia odora define a class of receptor-like proteins required for the normal accumulation of cuticular waxes. Plant Physiol **113:** 1091-1100

Hartmann U, Hohmann S, Nettesheim K, Wisman E, Saedler H, Huijser P (2000) Molecular cloning of SVP: a negative regulator of the floral transition in Arabidopsis. Plant Journal **21:** 351-360

Hawkins RD, Hon GC, Ren B (2010) Next-generation genomics: an integrative approach. Nat Rev Genet **11:** 476-486

Heck GR, Perry SE, Nichols KW, Fernandez DE (1995) AGL15, a MADS domain protein expressed in developing embryos. Plant Cell **7:** 1271-1282

Hegedus D, Yu M, Baldwin D, Gruber M, Sharpe A, Parkin I, Whitwill S, Lydiate D (2003) Molecular characterization of Brassica napus NAC domain transcriptional activators induced in response to biotic and abiotic stress. Plant Molecular Biology **53:** 383-397

Heitz T, Bergey DR, Ryan CA (1997) A gene encoding a chloroplast-targeted lipoxygenase in tomato leaves is transiently induced by wounding, systemin, and methyl jasmonate. Plant Physiol **114:** 1085-1093

Hellens RP, Allan AC, Friel EN, Bolitho K, Grafton K, Templeton MD, Karunairetnam S, Gleave AP, Laing WA (2005) Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Plant Methods **1:** 13

Henschel K, Kofuji R, Hasebe M, Saedler H, Munster T, Theissen G (2002) Two ancient classes of MIKC-type MADS-box genes are present in the moss Physcomitrella patens. Molecular Biology and Evolution **19:** 801-814

Hileman LC, Sundstrom JF, Litt A, Chen MQ, Shumba T, Irish VF (2006) Molecular and phylogenetic analyses of the MADS-Box gene family in tomato. Molecular Biology and Evolution **23:** 2245-2258

Hill TA, Day CD, Zondlo SC, Thackeray AG, Irish VF (1998) Discrete spatial and temporal cis-acting elements regulate transcription of the Arabidopsis floral homeotic gene APETALA3. Development **125:** 1711-1721

Ho LC, Grange RI, Picken AJ (1987) An Analysis of the Accumulation of Water and Dry-Matter in Tomato Fruit. Plant Cell and Environment **10:** 157-162

Hobson GE, Nichols, R., Davies, J.N., Atkey, P.T. (1984) The inhibition of tomato fruit ripening by silver. J Plant Physiol **116:** 21-29

Hong RL, Hamaguchi L, Busch MA, Weigel D (2003) Regulatory elements of the floral homeotic gene AGAMOUS identified by phylogenetic footprinting and shadowing. Plant Cell **15:** 1296-1309

Honma T, Goto K (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature **409:** 525-529

Huijser P, Klein J, Lonnig WE, Meijer H, Saedler H, Sommer H (1992) Bracteomania, an inflorescence anomaly, is caused by the loss of function of the MADS-box gene squamosa in Antirrhinum majus. Embo J **11:** 1239-1249

Immink RGH, Tonaco IAN, de Folter S, Shchennikova A, van Dijk ADJ, Busscher-Lange J, Borst JW, Angenent GC (2009) SEPALLATA3: the 'glue' for MADS box transcription factor complex formation. Genome Biology **10:** -

Islam MA, Du H, Ning J, Ye H, Xiong L (2009) Characterization of Glossy1-homologous genes in rice involved in leaf wax accumulation and drought resistance. Plant Mol Biol **70:** 443-456

Itkin M, Seybold H, Breitel D, Rogachev I, Meir S, Aharoni A (2009) The TOMATO AGAMOUS-LIKE 1 is a Component of the Fruit Ripening Regulatory Network. Plant J

Ito Y, Kitagawa M, Ihashi N, Yabe K, Kimbara J, Yasuda J, Ito H, Inakuma T, Hiroi S, Kasumi T (2008) DNA-binding specificity, transcriptional activation potential, and the rin mutation effect for the tomato fruit-ripening regulator RIN. Plant J **55:** 212-223

Jaakola L, Poole M, Jones MO, Kamarainen-Karppinen T, Koskimaki JJ, Hohtola A, Haggman H, Fraser PD, Manning K, King GJ, Thomson H, Seymour GB (2010) A SQUAMOSA MADS-box gene involved in the regulation of anthocyanin accumulation in bilberry fruits. Plant Physiol

Jones B, Frasse P, Olmos E, Zegzouti H, Li ZG, Latche A, Pech JC, Bouzayen M (2002) Down-regulation of DR12, an auxin-response-factor homolog, in the tomato results in a pleiotropic phenotype including dark green and blotchy ripening fruit. Plant Journal **32:** 603-613

Kalua CM, Boss PK (2009) Evolution of volatile compounds during the development of cabernet sauvignon grapes (Vitis vinifera L.). J Agric Food Chem **57:** 3818-3830

Kaufmann K, Melzer R, Theissen G (2005) MIKC-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. Gene **347:** 183-198

Kaufmann K, Muino JM, Jauregui R, Airoldi CA, Smaczniak C, Krajewski P, Angenent GC (2009) Target genes of the MADS transcription factor SEPALLATA3: integration of developmental and hormonal pathways in the Arabidopsis flower. PLoS Biol **7:** e1000090

Kaufmann K, Muino JM, Osteras M, Farinelli L, Krajewski P, Angenent GC (2006) Chromatin immunoprecipitation (ChIP) of plant transcription factors followed by sequencing (ChIP-SEQ) or hybridization to whole genome arrays (ChIP-CHIP). Nat Protoc **5:** 457-472

Kaufmann K, Wellmer F, Muino JM, Ferrier T, Wuest SE, Kumar V, Serrano-Mislata A, Madueno F, Krajewski P, Meyerowitz EM, Angenent GC, Riechmann JL (2003) Orchestration of floral initiation by APETALA1. Science **328:** 85-89

Kevany BM, Tieman DM, Taylor MG, Cin VD, Klee HJ (2007) Ethylene receptor degradation controls the timing of ripening in tomato fruit. Plant J **51:** 458-467

Kim SG, Kim SY, Park CM (2007) A membrane-associated NAC transcription factor regulates salt-responsive flowering via FLOWERING LOCUS T in Arabidopsis. Planta **226:** 647-654

Klann E, Yelle S, Bennett AB (1992) Tomato Fruit Acid Invertase Complementary-DNA - Nucleotide and Deduced Amino-Acid-Sequences. Plant Physiology **99:** 351-353

Klann EM, Hall B, Bennett AB (1996) Antisense acid invertase (TIV1) gene alters soluble sugar composition and size in transgenic tomato fruit. Plant Physiol **112:** 1321-1330

Kneissl ML, Deikman J (1996) The Tomato E8 Gene Influences Ethylene Biosynthesis in Fruit but Not in Flowers. Plant Physiol **112:** 537-547

Ko JH, Yang SH, Park AH, Lerouxel O, Han KH (2007) ANAC012, a member of the plant-specific NAC transcription factor family, negatively regulates xylary fiber development in Arabidopsis thaliana. Plant J **50:** 1035-1048

Kosma DK, Parsons EP, Isaacson T, Lu S, Rose JK, Jenks MA (2009) Fruit cuticle lipid composition during development in tomato ripening mutants. Physiol Plant **139:** 107-117

Kotilainen M, Elomaa P, Uimari A, Albert VA, Yu D, Teeri TH (2000) GRCD1, an AGL2-like MADS box gene, participates in the C function during stamen development in Gerbera hybrida. Plant Cell **12:** 1893-1902

Kovacs K, Fray RG, Tikunov Y, Graham N, Bradley G, Seymour GB, Bovy AG, Grierson D (2009) Effect of tomato pleiotropic ripening mutations on flavour volatile biosynthesis. Phytochemistry **70:** 1003-1008

Kramer EM, Dorit RL, Irish VF (1998) Molecular evolution of genes controlling petal and stamen development: Duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages. Genetics **149:** 765-783

Kramer EM, Dorit RL, Irish VF (1999) Molecular evolution of genes controlling petal and stamen development: Duplication and divergence within the APETALA3 and PISTILLATA MADS-box gene lineages (vol 149, pg 765, 1998). Genetics **151:** 915-915

Krizek BA, Meyerowitz EM (1996) The Arabidopsis homeotic genes APETALA3 and PISTILLATA are sufficient to provide the B class organ identity function. Development **122:** 11-22

Kutter C, Schob H, Stadler M, Meins F, Jr., Si-Ammour A (2007) MicroRNA-mediated regulation of stomatal development in Arabidopsis. Plant Cell **19:** 2417-2429

Lamb RS, Irish VF (2003) Functional divergence within the APETALA3/PISTILLATA floral homeotic gene lineages. Proceedings of the National Academy of Sciences of the United States of America **100:** 6558-6563

Larue CT, Wen J, Walker JC (2009) A microRNA-transcription factor module regulates lateral organ size and patterning in Arabidopsis. Plant J **58:** 450-463

Lee H, Suh SS, Park E, Cho E, Ahn JH, Kim SG, Lee JS, Kwon YM, Lee I (2000) The AGAMOUS-LIKE 20 MADS domain protein integrates floral inductive pathways in Arabidopsis. Genes & Development **14:** 2366-2376

Lee J, He K, Stolc V, Lee H, Figueroa P, Gao Y, Tongprasit W, Zhao H, Lee I, Deng XW (2007) Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. Plant Cell **19:** 731-749

Lelievre J-ML, A.; Jones, B; Bouzayen, M; Pech JC (1997) Ethylene and Fruit Ripening. Physiol Plant **110:** 727-739

Lelievre JM, Latche A, Jones B, Bouzayen M, Pech JC (1997) Ethylene and fruit ripening. Physiol Plant **101:** 727-739

Leon J, Rojo E, Sanchez-Serrano JJ (2001) Wound signalling in plants. J Exp Bot **52:** 1-9

Leonardi C, Baille A, Guichard S (1999) Effects of fruit characteristics and climatic conditions on tomato transpiration in a greenhouse. Journal of Horticultural Science & Biotechnology **74:** 748-756

Leone A, Bleve-Zacheo T, Gerardi C, Melillo MT, Leo L, Zacheo G (2006) Lipoxygenase involvement in ripening strawberry. J Agric Food Chem **54:** 6835-6844

Lin Z, Hong Y, Yin M, Li C, Zhang K, Grierson D (2008) A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening. Plant J **55:** 301-310

Lin Z, Zhong S, Grierson D (2009) Recent advances in ethylene research. J Exp Bot **60:** 3311-3336

Lincoln JE, Campbell AD, Oetiker J, Rottmann WH, Oeller PW, Shen NF, Theologis A (1993) LE-ACS4, a fruit ripening and wound-induced 1-aminocyclopropane-1-carboxylate synthase gene of tomato (Lycopersicon esculentum). Expression in Escherichia coli, structural characterization, expression characteristics, and phylogenetic analysis. J Biol Chem **268:** 19422-19430

Lincoln JE, Cordes S, Read E, Fischer RL (1987) Regulation of gene expression by ethylene during Lycopersicon esculentum (tomato) fruit development. Proc Natl Acad Sci U S A **84:** 2793-2797

Lincoln JE, Fischer RL (1988) Regulation of Gene Expression by Ethylene in Wild-Type and rin Tomato (Lycopersicon esculentum) Fruit. Plant Physiol **88:** 370-374

Lozano R, Angosto T, Gomez P, Payan C, Capel J, Huijser P, Salinas J, Martinez-Zapater JM (1998) Tomato flower abnormalities induced by low temperatures are associated with changes of expression of MADS-Box genes. Plant Physiol **117:** 91-100

Lozano R, Gimenez E, Cara B, Capel J, Angosto T (2009) Genetic analysis of reproductive development in tomato. Int J Dev Biol **53:** 1635-1648

Ma H, Yanofsky MF, Meyerowitz EM (1991) Agl1-Agl6, an Arabidopsis Gene Family with Similarity to Floral Homeotic and Transcription Factor Genes. Genes & Development **5:** 484-495

Mallory AC, Dugas DV, Bartel DP, Bartel B (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. Current Biology **14:** 1035-1046

Manning K, Tor M, Poole M, Hong Y, Thompson AJ, King GJ, Giovannoni JJ, Seymour GB (2006) A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nat Genet **38:** 948-952

Manzara T, Carrasco P, Gruissem W (1991) Developmental and organ-specific changes in promoter DNA-protein interactions in the tomato rbcS gene family. Plant Cell **3:** 1305-1316

Mapelli S, Lombardi L (1982) A Comparative Auxin and Cytokinin Study in Normal and to-2 Mutant Tomato Plants. Plant and Cell Physiology **23:** 751-757

Messenguy F, Dubois E (2003) Role of MADS box proteins and their cofactors in combinatorial control of gene expression and cell development. Gene **316:** 1-21

Michaels SD, Amasino RM (1999) FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11:** 949-956

Millerd A, Bonner J, Biale JB (1953) The Climacteric Rise in Fruit Respiration as Controlled by Phosphorylative Coupling. Plant Physiol **28:** 521-531

Mitsuda N, Iwase A, Yamamoto H, Yoshida M, Seki M, Shinozaki K, Ohme-Takagi M (2007) NAC transcription factors, NST1 and NST3, are key regulators of the formation of secondary walls in woody tissues of Arabidopsis. Plant Cell **19:** 270-280

Mitsuda N, Ohme-Takagi M (2008) NAC transcription factors NST1 and NST3 regulate pod shattering in a partially redundant manner by promoting secondary wall formation after the establishment of tissue identity. Plant J **56:** 768-778

Mitsuda N, Seki M, Shinozaki K, Ohme-Takagi M (2005) The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. Plant Cell **17:** 2993-3006

Montgomery J, Pollard V, Deikman J, Fischer RL (1993) Positive and negative regulatory regions control the spatial distribution of polygalacturonase transcription in tomato fruit pericarp. Plant Cell **5:** 1049-1062

Moxon S, Jing R, Szittya G, Schwach F, Rusholme Pilcher RL, Moulton V, Dalmay T (2008) Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening. Genome Res **18:** 1602-1609

Mueller LA, Solow TH, Taylor N, Skwarecki B, Buels R, Binns J, Lin C, Wright MH, Ahrens R, Wang Y, Herbst EV, Keyder ER, Menda N, Zamir D, Tanksley SD (2005) The SOL Genomics Network: a comparative resource for Solanaceae biology and beyond. Plant Physiol **138:** 1310-1317

Nakashima K, Tran LSP, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant Journal **51:** 617-630

Nakatsuka A, Murachi S, Okunishi H, Shiomi S, Nakano R, Kubo Y, Inaba A (1998) Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. Plant Physiol **118:** 1295-1305

Ng M, Yanofsky MF (2001) Function and evolution of the plant MADS-box gene family. Nat Rev Genet **2:** 186-195

Nicholass FJ, Smith CJ, Schuch W, Bird CR, Grierson D (1995) High levels of ripening-specific reporter gene expression directed by tomato fruit polygalacturonase gene-flanking regions. Plant Mol Biol **28:** 423-435

Norman C, Runswick M, Pollock R, Treisman R (1988) Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell **55:** 989-1003

Oetiker JH, Olson DC, Shiu OY, Yang SF (1997) Differential induction of seven 1-aminocyclopropane-1-carboxylate synthase genes by elicitor in suspension cultures of tomato (Lycopersicon esculentum). Plant Mol Biol **34:** 275-286

Ogo Y, Kobayashi T, Nakanishi Itai R, Nakanishi H, Kakei Y, Takahashi M, Toki S, Mori S, Nishizawa NK (2008) A novel NAC transcription factor, IDEF2, that recognizes the iron deficiency-responsive element 2 regulates the genes involved in iron homeostasis in plants. J Biol Chem **283:** 13407-13417

Oh SK, Lee S, Yu SH, Choi D (2005) Expression of a novel NAC domain-containing transcription factor (CaNAC1) is preferentially associated with incompatible interactions between chili pepper and pathogens. Planta **222:** 876-887

Ohnishi T, Sugahara S, Yamada T, Kikuchi K, Yoshiba Y, Hirano HY, Tsutsumi N (2005) OsNAC6, a member of the NAC gene family, is induced by various stresses in rice. Genes & Genetic Systems **80:** 135-139

Olsen AN, Ernst HA, Leggio LL, Skriver K (2005) NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci **10:** 79-87

Olsen AN, Ernst HA, Lo Leggio L, Johansson E, Larsen S, Skriver K (2004) Preliminary crystallographic analysis of the NAC domain of ANAC, a member of the plant-specific NAC transcription factor family. Acta Crystallogr D Biol Crystallogr **60:** 112-115

Olsen AN, Ernst HA, Lo Leggio L, Skriver K (2005) DNA-binding specificity and molecular functions of NAC transcription factors. Plant Science **169:** 785-797

Olson DC, White JA, Edelman L, Harkins RN, Kende H (1991) Differential expression of two genes for 1-aminocyclopropane-1-carboxylate synthase in tomato fruits. Proc Natl Acad Sci U S A **88:** 5340-5344

Ooka H, Satoh K, Doi K, Nagata T, Otomo Y, Murakami K, Matsubara K, Osato N, Kawai J, Carninci P, Hayashizaki Y, Suzuki K, Kojima K, Takahara Y, Yamamoto K, Kikuchi S (2003) Comprehensive analysis of NAC family genes in Oryza sativa and Arabidopsis thaliana. DNA Res **10:** 239-247

Orfila C, Huisman MM, Willats WG, van Alebeek GJ, Schols HA, Seymour GB, Knox JP (2002) Altered cell wall disassembly during ripening of Cnr tomato fruit: implications for cell adhesion and fruit softening. Planta **215:** 440-447

Orzaez D, Granell A (2009) Reverse genetics and transient gene expression in fleshy fruits: overcoming plant stable transformation. Plant Signal Behav **4:** 864-867

Orzaez D, Medina A, Torre S, Fernandez-Moreno JP, Rambla JL, Fernandez-Del-Carmen A, Butelli E, Martin C, Granell A (2009) A visual reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation. Plant Physiol **150:** 1122-1134

Orzaez D, Mirabel S, Wieland WH, Granell A (2006) Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit. Plant Physiol **140:** 3-11

Parenicova L, de Folter S, Kieffer M, Horner DS, Favalli C, Busscher J, Cook HE, Ingram RM, Kater MM, Davies B, Angenent GC, Colombo L (2003)

Molecular and phylogenetic analyses of the complete MADS-box transcription factor family in Arabidopsis: New openings to the MADS world. Plant Cell **15:** 1538-1551

Passmore S, Elble R, Tye BK (1989) A Protein Involved in Minichromosome Maintenance in Yeast Binds a Transcriptional Enhancer Conserved in Eukaryotes. Genes & Development **3:** 921-935

Payton S, Fray RG, Brown S, Grierson D (1996) Ethylene receptor expression is regulated during fruit ripening, flower senescence and abscission. Plant Mol Biol **31:** 1227-1231

Pecker I, Chamovitz D, Linden H, Sandmann G, Hirschberg J (1992) A single polypeptide catalyzing the conversion of phytoene to zeta-carotene is transcriptionally regulated during tomato fruit ripening. Proc Natl Acad Sci U S A **89:** 4962-4966

Pelaz S, Gustafson-Brown C, Kohalmi SE, Crosby WL, Yanofsky MF (2001) APETALA1 and SEPALLATA3 interact to promote flower development. Plant Journal **26:** 385-394

Pellegrini L, Song T, Richmond TJ (1995) Structure of Serum Response Factor Core Bound to DNA. Nature **376:** 490-498

Penarrubia L, Aguilar M, Margossian L, Fischer RL (1992) An Antisense Gene Stimulates Ethylene Hormone Production during Tomato Fruit Ripening. Plant Cell **4:** 681-687

Perry SE, Nichols KW, Fernandez DE (1996) The MADS domain protein AGL15 localizes to the nucleus during early stages of seed development. Plant Cell **8:** 1977-1989

Picton S, Gray JE, Payton S, Barton SL, Lowe A, Grierson D (1993) A histidine decarboxylase-like mRNA is involved in tomato fruit ripening. Plant Mol Biol **23:** 627-631

Pierrou S, Enerback S, Carlsson P (1995) Selection of High-Affinity Binding-Sites for Sequence-Specific, DNA-Binding Proteins from Random Sequence Oligonucleotides. Analytical Biochemistry **229:** 99-105

Plaut Z, Grava A, Yehezkel C, Matan E (2004) How do salinity and water stress affect transport of water, assimilates and ions to tomato fruits? Physiologia Plantarum **122:** 429-442

Pnueli L, Abu-Abeid M, Zamir D, Nacken W, Schwarz-Sommer Z, Lifschitz E (1991) The MADS box gene family in tomato: temporal expression during floral development, conserved secondary structures and homology with homeotic genes from Antirrhinum and Arabidopsis. Plant J **1:** 255-266

Pnueli L, Hareven D, Rounsley SD, Yanofsky MF, Lifschitz E (1994) Isolation of the tomato AGAMOUS gene TAG1 and analysis of its homeotic role in transgenic plants. Plant Cell **6:** 163-173

Prasanna V, Prabha TN, Tharanathan RN (2007) Fruit ripening phenomena--an overview. Crit Rev Food Sci Nutr **47:** 1-19

Raman S, Greb T, Peaucelle A, Blein T, Laufs P, Theres K (2008) Interplay of miR164, CUP-SHAPED COTYLEDON genes and LATERAL SUPPRESSOR controls axillary meristem formation in Arabidopsis thaliana. Plant J **55:** 65-76

Ray J, Moureau P, Bird C, Bird A, Grierson D, Maunders M, Truesdale M, Bramley P, Schuch W (1992) Cloning and characterization of a gene involved in phytoene synthesis from tomato. Plant Mol Biol **19:** 401-404

Ren T, Qu F, Morris TJ (2000) HRT gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to turnip crinkle virus. Plant Cell **12:** 1917-1926

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science **290:** 2105-2110

Riechmann JL, Meyerowitz EM (1997) Determination of floral organ identity by Arabidopsis MADS domain homeotic proteins AP1, AP3, PI, and AG is independent of their DNA-binding specificity. Molecular Biology of the Cell **8:** 1243-1259

Riechmann JL, Meyerowitz EM (1997) MADS domain proteins in plant development. Biol Chem **378:** 1079-1101

Riechmann JL, Wang MQ, Meyerowitz EM (1996) DNA-binding properties of Arabidopsis MADS domain homeotic proteins APETALA1, APETALA3, PISTILLATA and AGAMOUS. Nucleic Acids Research **24:** 3134-3141

Robertson M (2004) Two transcription factors are negative regulators of gibberellin response in the HvSPY-signaling pathway in barley aleurone. Plant Physiol **136:** 2747-2761

Robles P, Pelaz S (2005) Flower and fruit development in Arabidopsis thaliana. Int J Dev Biol **49:** 633-643

Rose JK, Cosgrove DJ, Albersheim P, Darvill AG, Bennett AB (2000) Detection of expansin proteins and activity during tomato fruit ontogeny. Plant Physiol **123:** 1583-1592

Rosin FM, Hart JK, Van Onckelen H, Hannapel DJ (2003) Suppression of a vegetative MADS box gene of potato activates axillary meristem development. Plant Physiol **131:** 1613-1622

Roslan HA, Salter MG, Wood CD, White MR, Croft KP, Robson F, Coupland G, Doonan J, Laufs P, Tomsett AB, Caddick MX (2001) Characterization of the ethanol-inducible alc gene-expression system in Arabidopsis thaliana. Plant J **28:** 225-235

Rottmann WH, Peter GF, Oeller PW, Keller JA, Shen NF, Nagy BP, Taylor LP, Campbell AD, Theologis A (1991) 1-aminocyclopropane-1-carboxylate synthase in tomato is encoded by a multigene family whose transcription is induced during fruit and floral senescence. J Mol Biol **222:** 937-961

Rushton PJ, Somssich IE, Ringler P, Shen QJ (2010) WRKY transcription factors. Trends Plant Sci **15:** 247-258

Sablowski RW, Meyerowitz EM (1998) A homolog of NO APICAL MERISTEM is an immediate target of the floral homeotic genes APETALA3/PISTILLATA. Cell **92:** 93-103

Saedler H, Huijser P (1993) Molecular biology of flower development in Antirrhinum majus (snapdragon). Gene **135:** 239-243

Saltveit ME, Dilley DR (1978) Rapidly Induced Wound Ethylene from Excised Segments of Etiolated Pisum sativum L., cv. Alaska: III. Induction and Transmission of the Response. Plant Physiol **62:** 710-712

Samalova M, Brzobohaty B, Moore I (2005) pOp6/LhGR: a stringently regulated and highly responsive dexamethasone-inducible gene expression system for tobacco. Plant J **41:** 919-935

Schwarz-Sommer Z, Hue I, Huijser P, Flor PJ, Hansen R, Tetens F, Lonnig WE, Saedler H, Sommer H (1992) Characterization of the Antirrhinum floral homeotic MADS-box gene deficiens: evidence for DNA binding and autoregulation of its persistent expression throughout flower development. EMBO J **11:** 251-263

Seki M, Ishida J, Narusaka M, Fujita M, Nanjo T, Umezawa T, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression pattern of around 7,000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. Funct Integr Genomics **2:** 282-291

Selth LA, Dogra SC, Rasheed MS, Healy H, Randles JW, Rezaian MA (2005) A NAC domain protein interacts with tomato leaf curl virus replication accessory protein and enhances viral replication. Plant Cell **17:** 311-325

Seo PJ, Kim MJ, Park JY, Kim SY, Jeon J, Lee YH, Kim J, Park CM (2010) Cold activation of a plasma membrane-tethered NAC transcription factor induces a pathogen resistance response in Arabidopsis. Plant Journal **61:** 661-671

Seymour G.B TJE, Tucker G.A., (1993) Biochemistry of Fruit Ripening, Ed 1st. Chapman & Hall, London

Seymour GB (1993) Biochemistry of Fruit Ripening. Chapman & Hall, London; New York

Sheldon CC, Burn JE, Perez PP, Metzger J, Edwards JA, Peacock WJ, Dennis ES (1999) The FLF MADS box gene: A repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell **11:** 445-458

Sheldon CC, Rouse DT, Finnegan EJ, Peacock WJ, Dennis ES (2000) The molecular basis of vernalization: The central role of FLOWERING LOCUS C (FLC).

Proceedings of the National Academy of Sciences of the United States of America **97:** 3753-3758

Shore P, Sharrocks AD (1995) The Mads-Box Family of Transcription Factors. European Journal of Biochemistry **229:** 1-13

Sieburth LE, Meyerowitz EM (1997) Molecular dissection of the AGAMOUS control region shows that cis elements for spatial regulation are located intragenically. Plant Cell **9:** 355-365

Solano R, Stepanova A, Chao Q, Ecker JR (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev **12:** 3703-3714

Sommer H, Beltran JP, Huijser P, Pape H, Lonnig WE, Saedler H, Schwarzsommer Z (1990) Deficiens, a Homeotic Gene Involved in the Control of Flower Morphogenesis in Antirrhinum-Majus - the Protein Shows Homology to Transcription Factors. Embo Journal **9:** 605-613

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R (1996) The no apical meristem gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell **85:** 159-170

Stepanova AN, Ecker JR (2000) Ethylene signaling: from mutants to molecules. Curr Opin Plant Biol **3:** 353-360

Sweetman JP, Chu C, Qu N, Greenland AJ, Sonnewald U, Jepson I (2002) Ethanol vapor is an efficient inducer of the alc gene expression system in model and crop plant species. Plant Physiol **129:** 943-948

Takada S, Hibara K, Ishida T, Tasaka M (2001) The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. Development **128:** 1127-1135

Tan S, Richmond TJ (1998) Crystal structure of the yeast MAT alpha 2/MCM1/DNA ternary complex. Nature **391:** 660-666

Tang WN, Perry SE (2003) Binding site selection for the MADS-domain protein AGL15: an in vitro and in vivo study. Faseb Journal **17:** A185-A185

Tanksley SD, McCouch SR (1997) Seed banks and molecular maps: unlocking genetic potential from the wild. Science **277:** 1063-1066

Taoka K, Yanagimoto Y, Daimon Y, Hibara K, Aida M, Tasaka M (2004) The NAC domain mediates functional specificity of CUP-SHAPED COTYLEDON proteins. Plant J **40:** 462-473

Theissen G (2001) Development of floral organ identity: stories from the MADS house. Current Opinion in Plant Biology **4:** 75-85

Theissen G, Becker A, Di Rosa A, Kanno A, Kim JT, Munster T, Winter KU, Saedler H (2000) A short history of MADS-box genes in plants. Plant Molecular Biology **42:** 115-149

Theissen G, Becker A, Di Rosa A, Kanno A, Kim JT, Munster T, Winter KU, Saedler H (2000) A short history of MADS-box genes in plants. Plant Mol Biol **42:** 115-149

Thomine S, Lelievre F, Debarbieux E, Schroeder JI, Barbier-Brygoo H (2003) AtNRAMP3, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. Plant J **34:** 685-695

Tieman DM, Ciardi JA, Taylor MG, Klee HJ (2001) Members of the tomato LeEIL (EIN3-like) gene family are functionally redundant and regulate ethylene responses throughout plant development. Plant J **26:** 47-58

Tieman DM, Klee HJ (1999) Differential expression of two novel members of the tomato ethylene-receptor family. Plant Physiol **120:** 165-172

Trainotti L, Pavanello A, Casadoro G (2005) Different ethylene receptors show an increased expression during the ripening of strawberries: does such an increment imply a role for ethylene in the ripening of these non-climacteric fruits? J Exp Bot **56:** 2037-2046

Tran LS, Nakashima K, Sakuma Y, Osakabe Y, Qin F, Simpson SD, Maruyama K, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K (2007) Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the ERD1 gene in Arabidopsis. Plant J **49:** 46-63

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell **16:** 2481-2498

Trobner W, Ramirez L, Motte P, Hue I, Huijser P, Lonnig WE, Saedler H, Sommer H, Schwarz-Sommer Z (1992) GLOBOSA: a homeotic gene which interacts with DEFICIENS in the control of Antirrhinum floral organogenesis. EMBO J **11:** 4693-4704

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006) A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science **314:** 1298-1301

Van Ieperen W, Volkov VS, Van Meeteren U (2003) Distribution of xylem hydraulic resistance in fruiting truss of tomato influenced by water stress. J Exp Bot **54:** 317-324

Van Ieperen W, Volkov VS, Van Meeteren U (2003) Distribution of xylem hydraulic resistance in fruiting truss of tomato influenced by water stress. Journal of Experimental Botany **54:** 317-324

Van Leene J, Witters E, Inze D, De Jaeger G (2008) Boosting tandem affinity purification of plant protein complexes. Trends in Plant Science **13:** 517-520

Varga A, Bruinsma J (1966) Tomato. *In* CRC Handbook of Fruit Set and Development. CRC Press, Boca Raton, FL, pp 461-480

Vrebalov J, Pan IL, Arroyo AJ, McQuinn R, Chung M, Poole M, Rose J, Seymour G, Grandillo S, Giovannoni J, Irish VF (2009) Fleshy Fruit Expansion and Ripening Are Regulated by the Tomato SHATTERPROOF Gene TAGL1. Plant Cell **21:** 3041-3062

Vrebalov J, Ruezinsky D, Padmanabhan V, White R, Medrano D, Drake R, Schuch W, Giovannoni J (2002) A MADS-box gene necessary for fruit ripening at the tomato ripening-inhibitor (rin) locus. Science **296:** 343-346

Vroemen CW, Mordhorst AP, Albrecht C, Kwaaitaal MA, de Vries SC (2003) The CUP-SHAPED COTYLEDON3 gene is required for boundary and shoot meristem formation in Arabidopsis. Plant Cell **15:** 1563-1577

Wang H, Tang W, Zhu C, Perry SE (2002) A chromatin immunoprecipitation (ChIP) approach to isolate genes regulated by AGL15, a MADS domain protein that preferentially accumulates in embryos. Plant J **32:** 831-843

Wang J, Chen G, Hu Z, Chen X (2007) Cloning and characterization of the EIN2-homology gene LeEIN2 from tomato. DNA Seq **18:** 33-38

Wang W, Vignani R, Scali M, Cresti M (2006) A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. Electrophoresis **27:** 2782-2786

Wang X, Goregaoker SP, Culver JN (2009) Interaction of the Tobacco Mosaic Virus Replicase Protein with a NAC Domain Transcription Factor Is Associated with the Suppression of Systemic Host Defenses. Journal of Virology **83:** 9720-9730

Weir I, Lu J, Cook H, Causier B, Schwarz-Sommer Z, Davies B (2004) CUPULIFORMIS establishes lateral organ boundaries in Antirrhinum. Development **131:** 915-922

West AG, Causier BE, Davies B, Sharrocks AD (1998) DNA binding and dimerisation determinants of Antirrhinum majus MADS-box transcription factors. Nucleic Acids Research **26:** 5277-5287

White PJ (2002) Recent advances in fruit development and ripening: an overview. J Exp Bot **53:** 1995-2000

Wilkinson JQ, Lanahan MB, Clark DG, Bleecker AB, Chang C, Meyerowitz EM, Klee HJ (1997) A dominant mutant receptor from Arabidopsis confers ethylene insensitivity in heterologous plants. Nat Biotechnol **15:** 444-447

Wilkinson JQ, Lanahan MB, Yen HC, Giovannoni JJ, Klee HJ (1995) An ethylene-inducible component of signal transduction encoded by never-ripe. Science **270:** 1807-1809

Windt CW, Gerkema E, Van As H (2009) Most Water in the Tomato Truss Is Imported through the Xylem, Not the Phloem: A Nuclear Magnetic Resonance Flow Imaging Study. Plant Physiology **151:** 830-842

Wyrick JJ, Young RA (2002) Deciphering gene expression regulatory networks. Curr Opin Genet Dev **12:** 130-136

Xie Q, Guo HS, Dallman G, Fang S, Weissman AM, Chua NH (2002) SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. Nature **419:** 167-170

Xie Q, Guo HS, Dallman G, Fang SY, Weissman AM, Chua NH (2002) SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. Nature **419:** 167-170

Xie Q, Sanz-Burgos AP, Guo H, Garcia JA, Gutierrez C (1999) GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. Plant Mol Biol **39:** 647-656

Xu R, Goldman S, Coupe S, Deikman J (1996) Ethylene control of E4 transcription during tomato fruit ripening involves two cooperative cis elements. Plant Mol Biol **31:** 1117-1127

Yabuta Y, Morishita T, Kojima Y, Maruta T, Nishizawa-Yokoi A, Shigeoka S (2010) Identification of recognition sequence of ANAC078 protein by the cyclic amplification and selection of targets technique. Plant Signal Behav **5**

Yalovsky S, Rodriguez-Concepcion M, Bracha K, Toledo-Ortiz G, Gruissem W (2000) Prenylation of the floral transcription factor APETALA1 modulates its function. Plant Cell **12:** 1257-1266

Yamaguchi M, Goue N, Igarashi H, Ohtani M, Nakano Y, Mortimer JC, Nishikubo N, Kubo M, Katayama Y, Kakegawa K, Dupree P, Demura T (2010) VASCULAR-RELATED NAC-DOMAIN6 (VND6) and VND7 Effectively Induce

Transdifferentiation into Xylem Vessel Elements under Control of an Induction System. Plant Physiol

Yamaguchi M, Kubo M, Fukuda H, Demura T (2008) Vascular-related NAC-DOMAIN7 is involved in the differentiation of all types of xylem vessels in Arabidopsis roots and shoots. Plant J **55:** 652-664

Yamaguchi M, Ohtani M, Mitsuda N, Kubo M, Ohme-Takagi M, Fukuda H, Demura T (2010) VND-INTERACTING2, a NAC Domain Transcription Factor, Negatively Regulates Xylem Vessel Formation in Arabidopsis. Plant Cell **22:** 1249-1263

Yang R, Deng C, Ouyang B, Ye Z (2010) Molecular analysis of two salt-responsive NAC-family genes and their expression analysis in tomato. Mol Biol Rep

Yang YW, Wu Y, Pirrello J, Regad F, Bouzayen M, Deng W, Li ZG (2010) Silencing Sl-EBF1 and Sl-EBF2 expression causes constitutive ethylene response phenotype, accelerated plant senescence, and fruit ripening in tomato. Journal of Experimental Botany **61:** 697-708

Yang YZ, Fanning L, Jack T (2003) The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, APETALA3 and PISTILLATA. Plant Journal **33:** 47-59

Yang YZ, Jack T (2004) Defining subdomains of the K domain important for protein-protein interactions of plant MADS proteins. Plant Molecular Biology **55:** 45-59

Yanofsky MF, Ma H, Bowman JL, Drews GN, Feldmann KA, Meyerowitz EM (1990) The Protein Encoded by the Arabidopsis Homeotic Gene Agamous Resembles Transcription Factors. Nature **346:** 35-39

Yao J, Dong Y, Morris BA (2001) Parthenocarpic apple fruit production conferred by transposon insertion mutations in a MADS-box transcription factor. Proc Natl Acad Sci U S A **98:** 1306-1311

Yokotani N, Tamura S, Nakano R, Inaba A, Kubo Y (2003) Characterization of a novel tomato EIN3-like gene (LeEIL4). J Exp Bot **54:** 2775-2776

Yokotani N, Tamura S, Nakano R, Inaba A, McGlasson WB, Kubo Y (2004) Comparison of ethylene- and wound-induced responses in fruit of wild-type, rin and nor tomatoes. Postharvest Biology and Technology **32:** 247-252

Yoshida H, Wang KL, Chang CM, Mori K, Uchida E, Ecker JR (2006) The ACC synthase TOE sequence is required for interaction with ETO1 family proteins and destabilization of target proteins. Plant Mol Biol **62:** 427-437

Yoshii M, Yamazaki M, Rakwal R, Kishi-Kaboshi M, Miyao A, Hirochika H (2010) The NAC transcription factor RIM1 of rice is a new regulator of jasmonate signaling. Plant Journal **61:** 804-815

Zhang B, Yin XR, Li X, Yang SL, Ferguson IB, Chen KS (2009) Lipoxygenase gene expression in ripening kiwifruit in relation to ethylene and aroma production. J Agric Food Chem **57:** 2875-2881

Zhang JZ (2003) Overexpression analysis of plant transcription factors. Current Opinion in Plant Biology **6:** 430-440

Zhao C, Craig JC, Petzold HE, Dickerman AW, Beers EP (2005) The xylem and phloem transcriptomes from secondary tissues of the Arabidopsis root-hypocotyl. Plant Physiol **138:** 803-818

Zhao L, Kim Y, Dinh TT, Chen X (2007) miR172 regulates stem cell fate and defines the inner boundary of APETALA3 and PISTILLATA expression domain in Arabidopsis floral meristems. Plant J **51:** 840-849

Zhao Q, Gallego-Giraldo L, Wang H, Zeng Y, Ding SY, Chen F, Dixon RA (2010) An NAC transcription factor orchestrates multiple features of cell wall development in Medicago truncatula. Plant J

Zheng Y, Ren N, Wang H, Stromberg AJ, Perry SE (2009) Global identification of targets of the Arabidopsis MADS domain protein AGAMOUS-Like15. Plant Cell **21:** 2563-2577

Zhong R, Demura T, Ye ZH (2006) SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. Plant Cell **18:** 3158-3170

Zhong R, Richardson EA, Ye ZH (2007) Two NAC domain transcription factors, SND1 and NST1, function redundantly in regulation of secondary wall synthesis in fibers of Arabidopsis. Planta **225:** 1603-1611

Zhou D, Kalaitzis P, Mattoo AK, Tucker ML (1996) The mRNA for an ETR1 homologue in tomato is constitutively expressed in vegetative and reproductive tissues. Plant Mol Biol **30:** 1331-1338

Zhu QH, Upadhyaya NM, Gubler F, Helliwell CA (2009) Over-expression of miR172 causes loss of spikelet determinacy and floral organ abnormalities in rice (Oryza sativa). BMC Plant Biol **9:** 149